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APL-TDR-64-70
Part II

MECHANISM OF MICROBIOLOGICAL CONTAMINATION OF JET FUEL AND DEVELOPMENT OF TECHNIQUES FOR DETECTION OF MICROBIOLOGICAL CONTAMINATION

Part II. The Role of Microorganisms in Aluminum Corrosion, Emulsion Formation, Sludge Formation and Sealant Degradation

TECHNICAL DOCUMENTARY REPORT NO. APL-TDR-64-70, PART II

February 1966

AF Aero Propulsion Laboratory
Research and Technology Division
Air Force Systems Command
Wright-Patterson Air Force Base, Ohio

Project No. 3048, Task No. 304801

(Prepared under Contract No. AF 33(657)-9186 by Melpar, Inc., a Subsidiary of Westinghouse Air Brake Company, Falls Church, Virginia; Gordon C. Blanchard and Charles R. Goucher, authors)

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Air Force Aero Propulsion Laboratory
Research and Technology Division
Air Force Systems Command
Wright-Patterson Air Force Base, Ohio

FOREWORD

This is Part II of the first interim summary report prepared under contract AF 33(657)-9186, "Mechanism of Microbiological Contamination of Jet Fuels and Development of Techniques for Detection of Microbiological Contamination." This contract was administered under the direction of the Air Force Aero Propulsion Laboratory, Research and Technology Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio; Mr. Jack Fultz, project engineer.

This report covers work conducted from 1 January 1964 to 31 December 1964.

ABSTRACT

Progress has been made in the past year in defining the role that microorganisms can play in the processes of aluminum alloy corrosion, emulsion formation, sludge formation, and sealant and topcoat degradation during the oxidation of jet fuel hydrocarbons for growth. It was demonstrated that bacteria can cause aerobic aluminum alloy corrosion by changing the ionic composition of the growth medium, by growth in proteinaceous media, and by bringing about corrosion in media containing electron mediators coupled to cell metabolism. Fractionation of growth media made corrosive by microorganisms growing on fuel has yielded two particularly corrosive fractions; one fraction contains cations and the other fraction contains colored organic compounds. Fungi caused corrosion by concentrating corrosive metals, i.e., copper and iron, in their mycelium. This metal reacted with aluminum when organisms were deposited upon it. Fungi also caused corrosion by their ability to penetrate coatings, thus exposing the aluminum to corrosive growth media. Three different metabolic products potentially capable of clogging filters were formed by microorganisms growing on jet fuel. One product formed a layer on top of the water phase and penetrated the fuel phase and could not be centrifuged out of solution. A second product classified as a "sludge" was heavier than water and accumulated in the water bottom phase of the jet fuel-water system. The third product caused an emulsion in the water layer. Unsaturated short-chain hydrocarbons including pentene, hexene, heptene, octene, and nonene (but not decene or dodecene) inhibited respiration and killed fuel isolates. The saturated homologs of these compounds were either innocuous or supported growth. Some fuel additives were observed to support the growth of fuel isolates while most anti-icing and metal deactivators were mildly toxic. Similar findings were observed concerning sealant and topcoat utilization by jet fuel microorganisms; some stimulated growth while others were inhibitory.

This technical report has been reviewed and is approved.

for Arthur V. Churchill, Chief
Fuels, Lubrication and Hazards Branch
Support Technology Division

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I. INTRODUCTION

This research has been undertaken in an attempt to determine the mechanism by which microorganisms cause operational difficulties in jet fuel systems. Some of these difficulties are represented by the corrosion of aluminum alloy jet fuel tanks. Other difficulties arise through the formation of sludges and emulsions caused by the metabolic activity of microorganisms contaminating fuel systems. Still other problems derive from the action of microorganisms on inorganic compounds which are contained in the ubiquitous fuel water bottom.

The first year of this study was devoted principally to determining the nature of fuel contaminants. Alterations in the microbial ecology of fuel-water systems were followed and the findings were related to chemical changes in those systems. Knowledge was obtained on how to efficiently grow organisms isolated from fuel, and experimental work was accomplished on methods for detecting microorganisms in fuel systems. Research in the second year, which is summarized in this report, defines problems of microbial fuel contamination in terms of the reactions of extraneous inorganic material in fuel-water systems and in terms of the production of corrosive compounds by the microorganisms found in fuel.

The research in the second year dealt with the operation of several chemical and biological mechanisms that permit microorganisms to form emulsions and cause aluminum corrosion. The enzymes catalyzing the oxidation of hydrocarbons were studied. The growth of fuel isolates on jet fuels in pure culture and mixed culture was followed for long periods of time. Corrosive compounds produced by microorganisms were obtained and characterized chromatographically and spectrophotometrically. The objective of the research in this period was to experimentally and conceptually segregate the many reactions associated with microbial growth that contribute to the corrosion of aluminum wing tanks and the chemical contamination of jet fuel. Studies were made of the conditions of environment which bring about aluminum corrosion by microorganisms. The hypothesis was proposed and tested that microbial growth results in the removal of naturally occurring inhibitors of fuel-water systems and leaves unused corrosive concentrations of elements such as iron, calcium, and chlorine. The alternate hypothesis that organisms produce corrosive compounds was tested. The hypothesis appeared to be partially proven by the ability of microbial cells to cause corrosion when grown in media containing high concentrations of nitrate.

Intermediary metabolism and respiratory mechanism of hydrocarbon-oxidizing organisms were studied. The results of these studies appear to justify the belief that a large measure of control can be exercised over the production of microbial fuel contaminants by strictly chemical means. It is believed that knowledge of biochemical and physiological activities of fuel-utilising microorganisms is required to arrive at a more rigorous definition of the problem of fuel contamination.

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II. SUMMARY

The research program for the past year has been concerned with determining the mechanism by which fuel-metabolizing microorganisms cause aluminum alloy corrosion, sludge formation, hydrocarbon oxidation, and degradation of sealants and topcoats.

The corrosion studies have been confined to the four hypothesis by which microorganisms could bring about aluminum corrosion.[1] The first hypothesis proposed that microorganisms cause corrosion by altering the relative concentration of biologically essential ions in a growth medium. The results of this effort showed that when bacteria remove phosphate and nitrate from a growth-supporting medium, the medium becomes more corrosive to aluminum. It was concluded that the greater corrosivity resulted from increasing the proportion of iron and calcium present in the medium. It was stressed: (1) that media supporting the growth and multiplication of microorganisms were intrinsically corrosive, and (2) that microorganisms can remove corrosion inhibitors such as nitrates or phosphate and cause the medium to become actively corrosive.

A second hypothesis was proposed and tested; i.e., the hypothesis that microorganisms can produce corrosive materials from the oxidation and transformation of hydrocarbon substrates. The actual production of such corrosive material was suggested by the corrosion of aluminum caused by microbial growth in a casein hydrolysate medium. This corrosion was not prevented by nitrate, and mineral constituents such as iron or calcium did not appear to stimulate it.

In further support of this second hypothesis, it was found that organisms also produce corrosive compounds when grown in mineral media for long periods of time. The time required for the production of these compounds was sometimes as great as 200 days. Corrosive, insoluble sludge was produced by cultures initially low in nitrate, and soluble, corrosive compounds were produced in cultures initially high in nitrate. Corrosion caused by the latter compounds was not prevented by the addition of nitrate.

J. Takahashi et al.[2] and K. Yamada et al.[3] report that microorganisms excrete protein and protein-like material into growth media during hydrocarbon oxidation. It may be supposed that water bottoms also contain organic contaminants; an investigation of a corrosive mechanism in which these reactants participate was begun with products of microbial metabolism such as proteins, peptides, and amino acids. These products were shown to corrode aluminum over long periods of time, but when microorganisms isolated from fuel were inoculated into such media (with a jet fuel overlay) aluminum corrosion took place in large areas in a short time. The corrosion produced by microorganisms in these media containing proteinaceous materials was not inhibited by nitrate. This corrosion appears to be different in character and represents the action of an important mechanism which differs from that operative in strictly mineral media with hydrocarbon overlays. Mixed cultures

from Ramey have been used, but not actual water bottom. Corrosion by a water bottom obtained from a storage tank was inhibited, however, by nitrate, and it may have resulted from a depletion of natural inhibition by microbial growth. Another important finding was that aluminum alloy in an immiscible system, i.e., fuel and water, is per se a concentration cell. The chemical activity of this concentration cell can be influenced by water-soluble or fuel-soluble microbial products. This was dramatically shown with systems containing water and hydrocarbon (chloroform, octane, or dinitrophenol) whereby it appears that corrosion may be brought about by the direct oxidation of organic compounds at the aluminum surface.

Research was accomplished on a third hypothesis, i.e., that corrosion of aluminum is caused by microorganisms establishing microcenters of galvanic activity on metal surfaces. It was shown that pitting corrosion took place under holidays in the coating. These coating lesions were produced by degenerative changes in microbial culture media. It was concluded that local galvanic activity contributed to the local pitting observed.

Corrosion pits were also formed with fungi which had been allowed to concentrate copper and iron from a medium. Apparently this bound metal is capable of establishing galvanic activity when deposited on the aluminum alloy.

Attempts were made, in a fourth hypothesis, to determine the contribution of the mechanism described to the corrosion phenomena. It was theorized that microorganisms remove electrons from metal surfaces; but the demonstration of the direct oxidation and corrosion of aluminum by microorganisms was unsuccessful. However, when electron mediators such as methylene blue were added to microbial cultures, they caused the deposition of microbial material or metabolic products on aluminum surfaces. It was concluded that these agents brought about conditions which led to aluminum corrosion.

Distinct microbial sludges have been observed to form during the growth of microorganisms on jet fuel. The material referred to as sludge is dark brown to black and assumes several forms depending on the organisms or combinations of organisms used, and the conditions and time of growth. Some of the material which we call sludge is not readily soluble in water and in nonpolar solvents. This sludge is often found in the bottom of old cultures and, while it is easily dispersed in the medium, it quickly settles out of suspension. Other sludge material is suspended in the medium and, under centrifugation, it floats. A third form of microbial product resembling sludge in color is water soluble and easily adsorbed to anion exchange resins and, possibly to electropositive centers in metallic fuel systems. This latter compound interests us because it has been associated with corrosion in tests made at this laboratory.

Both microbial sludge and emulsions were analyzed chemically and found to contain, respectively, C, H, N, and O, as well as fatty acids. The C, H, N, and O, in certain chemical combinations, could produce corrosion and fatty acids could cause emulsion formation.

The effect of common fuel additives on the growth and survival of fuel isolates and on corrosion was studied. Some additives actively stimulated microbial growth while others were mildly toxic at high concentrations. Similar findings were also observed where sealants and topcoating materials were used as a sole course of carbon or nitrogen for microbial growth. In this case, the polyurethane and polysulfide coatings were least attacked by the microorganisms.

A study on the mechanism of hydrocarbon oxidation by fuel organisms was begun. It was believed that this study would furnish clues to the mechanism of emulsion formation and corrosion. Fuel isolates were found which were constitutively adapted to the oxidation of jet fuel hydrocarbons while others required a period of adaption to hydrocarbon oxidation. Fuel isolates oxidized jet fuel almost as rapidly as glucose, and were variable in their requirements for minerals to affect fuel oxidation. Acidic conditions occurred with fuel oxidation. Tests were made of the possibility of controlling the growth of fuel organisms by using respiratory inhibitors, but the respiration of fuel isolates was surprisingly resistant to azide and 2,4-dinitrophenol inhibition. But respiration of fuel isolates was stopped by 2-hexene or 1-heptene alone or when mixed with either glucose or fuel. In addition to preventing substrate oxidation, these unsaturated compounds were found to be biocidal. This suggested the possibility of designing water-soluble compounds with unsaturated hydrocarbon moieties for the control and study of metabolic fuel contamination processes.

III. FUTURE WORK

We have demonstrated some mechanisms by which microorganisms can cause corrosion, sludge and emulsion formation, fuel deterioration, and destruction of sealants and topcoats. The results of these studies show the effects induced by fuel microorganisms under rigorously controlled conditions and with the use of certain aluminum alloys. Thus, certain conditions permitting microbial corrosion of aluminum have been defined. Still, we have not approached this problem from the molecular level, and is believed that a need exists to determine which microbial products, or which of the chemical constituents react, and in what way, with aluminum surfaces.*

To obtain a better understanding of how microorganisms are performing corrosive chemical changes will require a more refined study--a study with the objective of elucidating microbial corrosion mechanisms in terms of the actual chemical entities produced by bacteria and capable of reaction with metals. Methods will be sought to detect the presence of microorganisms in consequence of these unique products. Also studies will be performed on rapid simple methods for detecting microorganisms in fuel-water bottoms with a sensitivity of 10^3 to 10^4 cells per ml. Consideration would be given to medium components including hydrocarbons, the growth conditions, and the extraction, isolation, and characterization of each product formed under various controlled conditions. The general feeling now is that the future studies should be carried out with pure hydrocarbons. This will make possible the quantitative balances of substrate used and products formed. In this way the relationship between hydrocarbon oxidation and each of the operational problems can be defined and the mechanism by which microorganisms cause the contamination or corrosion phenomena can be studied.

The corrosion studies will have as their objective the understanding of several possible mechanisms of microbial corrosion. One of the problems to be studied concerns how organisms utilize corrosion inhibitors, i.e., nitrate and phosphate, and others, from the medium and to determine quantitatively what products are formed from these compounds. First it would be important to know how the organism reduces nitrate and to determine what relationship this has to hydrocarbon oxidation. These processes must be energetically coupled and physiologically dependent. Work is now in progress to produce, purify, and characterize products formed from hydrocarbon oxidation. It is desired that effort be placed on concentration cell corrosion. Research would also be continued on the action of the microorganisms as concentration cells as well as research on those cells formed by immiscible solutions in contact with aluminum surfaces. The inhibition of respiration and killing caused by the short chain unsaturated hydrocarbons will be further studied with whole cells, cell-free systems, and purified enzymes. This information should provide clues to better methods for controlling organisms in fuel systems and should help in elucidating the enzyme systems responsible for the initial stages in hydrocarbon oxidation.

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*See Flow Diagram of Accomplishments and Future Plans, page 91.

The study of the composition of sludges and emulsions will continue and attempts will be made to compare the products formed on jet fuel with those formed on pure hydrocarbons. Most of the problems caused by microbial growth in fuel systems stem from the unique ability of fuel microorganisms to oxidize hydrocarbons; therefore, the chemical mechanism making these oxidations possible should be understood.

IV. EXPERIMENTAL WORK AND DISCUSSION

In this section the problem of microbial contamination of jet fuel is defined in terms of the metabolic action of microorganisms on fuel system components.

The causes of microbial corrosion of aluminum are discussed with respect to the ability of microorganisms to utilize corrosion inhibitors and produce corrosive compounds during jet fuel oxidation.

A more precise understanding of the biological or chemical mechanisms of fuel system contamination was obtained by purifying compounds synthesized from jet fuel by microorganisms. These compounds ranged in structure from simple lipid material to probable nitrated compounds of considerable complexity. These compounds were related to specific problems of fuel system contamination: cells high in lipids floated and formed water-jet fuel emulsions; media high in nitrated organic compounds were corrosive to aluminum alloys.

The biochemical activity of microbial fuel contaminants were also characterized. The organisms showed an unexpected resistance to known respiratory inhibitors such as azide, dinitrophenol, and EDTA.* The organisms showed an unexpected sensitivity to unsaturated hydrocarbons of medium chain length. These olefins both prevented respiration and killed the fuel isolates tested, but differ by but one double bond from hydrocarbons acting as carbon sources for growth.

The physical and chemical characteristics of cultures oxidizing jet fuel for long periods of time were related to filter clogging. Products formed in the aqueous phase in old cultures when placed in systems containing an immiscible organic solvent appeared to stimulate aluminum corrosion. The microbial deterioration of sealants and coatings was characterized and these materials were shown to contain compounds capable of supporting microbial growth.

A. Mechanisms of Aluminum Corrosion

The corrosion of aircraft wing tanks has been associated with the growth of microorganisms in jet fuel systems. But while the corrosion of metal by microorganisms has been a subject of sustained interest for many years, these studies dealt almost exclusively with corrosion of ferrous metals under anaerobic conditions. Yet wing tank corrosion takes place in the presence of oxygen and where the metal involved is aluminum alloy.

The ability of certain few microorganisms to cause ferrous metal corrosion in the absence of oxygen was attributed to their content of the enzyme hydrogenase and to their ability to reduce elemental sulfur with the formation

*Ethylene diamine tetra-acetic acid

of hydrogen sulfide. Hydrogenase catalyzed the oxidation of hydrogen gas adsorbed to metal surfaces and in this way brought about cathodic depolarization and corrosion. Hydrogen sulfide produced by microorganisms caused anodic depolarization by reacting with ferrous or ferric ions and caused corrosion. Through the operation of these two mechanisms microorganisms caused a destructive attack on ferrous metals.

In contrast to iron corrosion, aluminum corrosion appeared to be brought about by microorganisms possessing neither a hydrogenase nor producing hydrogen sulfide.* Thus in attempting to understand the mechanism of aerobic corrosion of nonferrous metals, four hypotheses were proposed and tested. These hypotheses were based on known chemical transformations that bring about the destruction of aluminum alloys and on known physiological activities of microorganisms. It was proposed that:

a. Microbial growth and metabolism causes changes in the mineral content of growth media and, by this means, microbes diminish the quantities of corrosion inhibitor present in water bottoms and cause corrosion.

b. Microbial metabolic products act as mediators in the corrosive process by stabilizing the oxidation reduction potential, or by complexing metal ions and thereby shifting the chemical equilibrium in favor of corrosion.

c. Microbial metabolism effects changes in the electrochemical properties of very confined areas such as those between microcolonies and metal or topcoats surfaces; thus centers of galvanic activity are established and corrosion results.

d. Microorganisms directly oxidize metal surfaces and cause the transfer of electrons from the metal to a physiological produced electron receptor.

Each of these propositions was tested during the previous year and each mechanism suggested appears to contribute in a different degree to the phenomenon of aerobic microbial corrosion of aluminum. The extent of this contribution depended on the growth phase of the organism, the mineral composition of the water medium suspending microorganisms, the organic composition of the suspending medium, and among many other variables, the composition of the aluminum alloys tested.

The salient results of the research effort of the past year concerned with aluminum corrosion are presented in this section.

1. Corrosion Caused by the Components of Bushnell-Haas Medium

The first studies of microbial corrosion of aluminum carried out were attempts to duplicate the work of others. Our studies, based on previous methods, indicated that a causal relationship between microbial growth and

*Under aerobic conditions.

aluminum corrosion could not be unequivocally established in the laboratory. Studies first referred to in the third quarterly report showed that the elimination of nitrate from a medium containing iron, calcium, magnesium, phosphate, ammonia, and sulfate caused the medium to become corrosive to aluminum alloys. This observation revealed that media often used for the growth of hydrocarbon-oxidizing organisms, like Bushnell-Haas medium, would have prevented the action of corrosive compounds should they have been produced by microorganisms; it would also inhibit the corrosive activity of ions frequently present in media supporting microbial growth.

To evaluate the contribution of microbial growth to aluminum corrosion, it was first necessary to determine the ability of the individual ions of the growth medium to cause corrosion in the absence of inhibitors such as nitrate and in the absence of microorganisms.

A preliminary screening of growth medium ions for ability to cause corrosion showed that both calcium salts and ferric salts caused extensive corrosion of the alloys 2024 and 7075 within short time periods. Corrosion by magnesium salts was variable and in general did not occur.

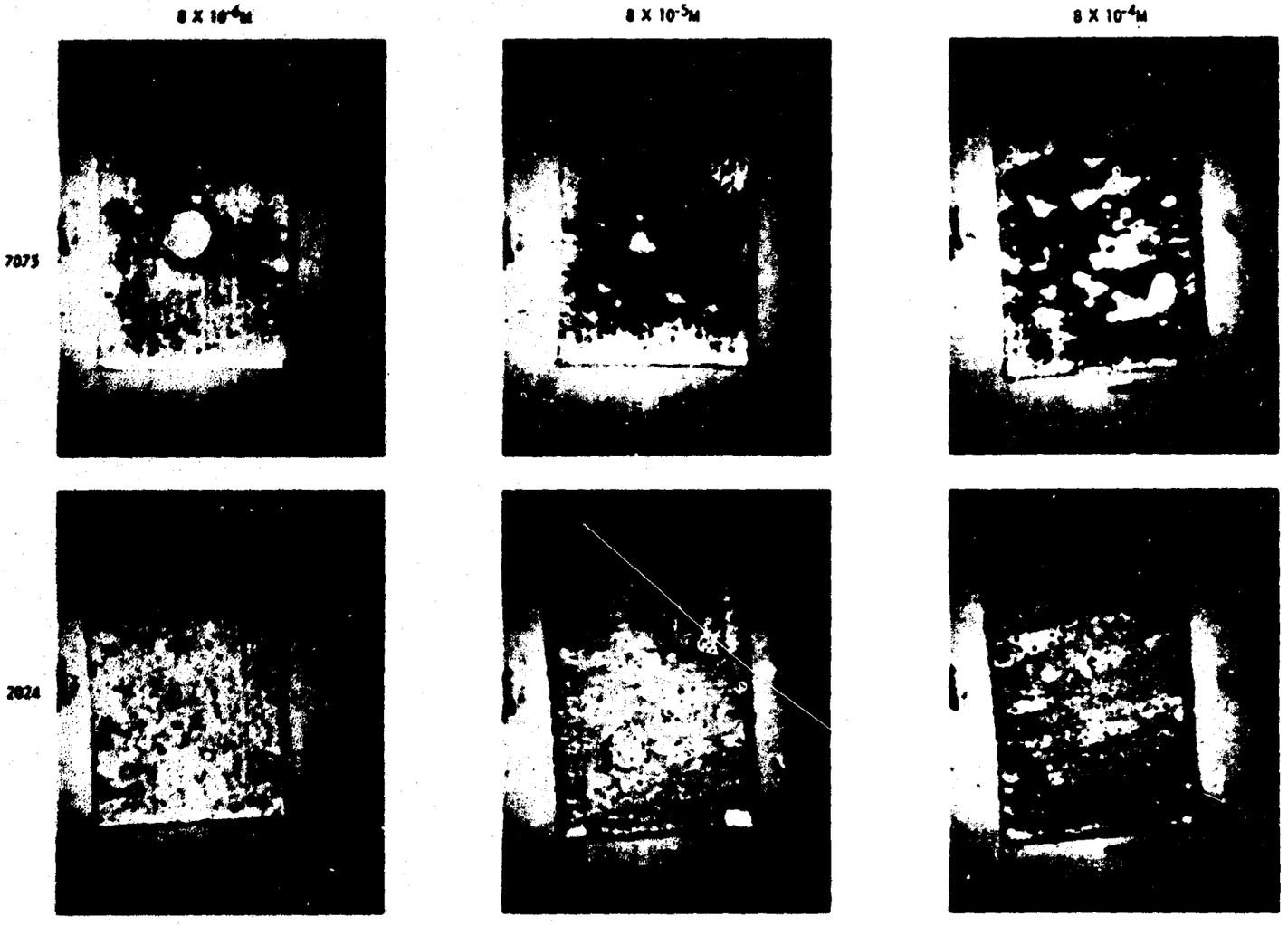
Tests for aluminum corrosion were made by submerging coupons of these alloys in aqueous test solution with a jet fuel overlay and placing the system on a rotary shaker at 30°C for variable lengths of time. Following this treatment, coupons were removed, rinsed carefully in distilled water, blotted dry, and photographed. The pictures in Figure 1 show the extent of corrosion to be a function of the concentration of ferric salt added. This pattern of aluminum corrosion in relationship to salt concentration is essentially the same as that observed with calcium sulfate, but the visible character of the corroded aluminum alloys is different with iron than with calcium. Other components of the Bushnell-Haas medium were tested by the same method used with calcium and iron. These inorganic components, potassium, orthophosphate, sulfate, and ammonia, did not cause corrosion.

2. Corrosion Inhibited by Bushnell-Haas Medium Components

Preliminary studies indicated that both phosphate and nitrate prevented the corrosion of aluminum caused by corrosive ions. Further studies were performed to establish the concentration levels of these inhibitors which were effective against various concentrations of substances which appear to stimulate aluminum corrosion.

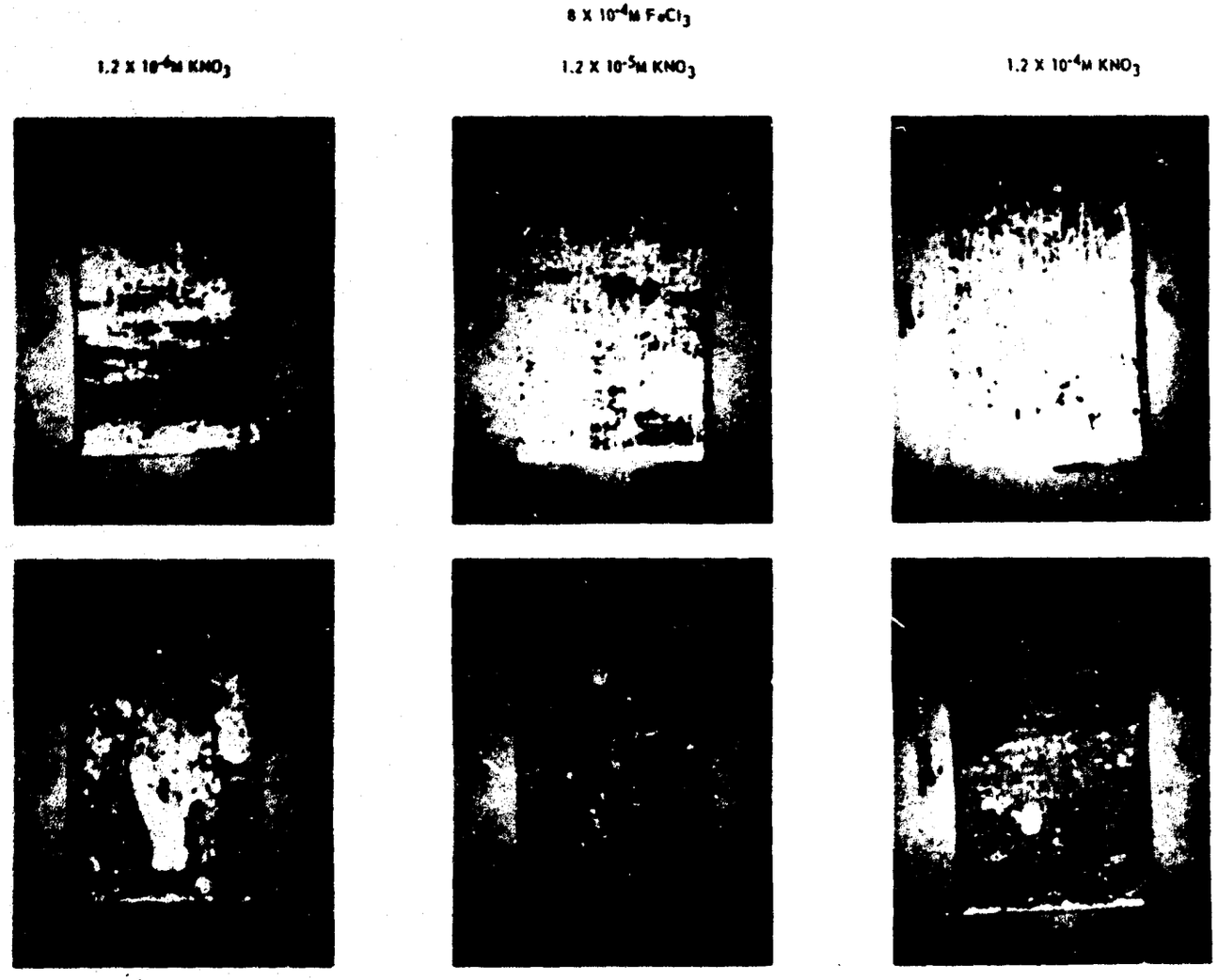
Tests were set up to determine the concentration of nitrate which would prevent the corrosion of aluminum in sterile solution caused by biologically essential ions at concentrations used in the Bushnell-Haas growth medium.

Figure 2 shows the inhibition of corrosion caused by 8×10^{-4} M $FeCl_3$ with various concentrations of nitrate. The half-maximum corrosion inhibition for the time interval observed was at 5.9×10^{-4} M KNO_3.



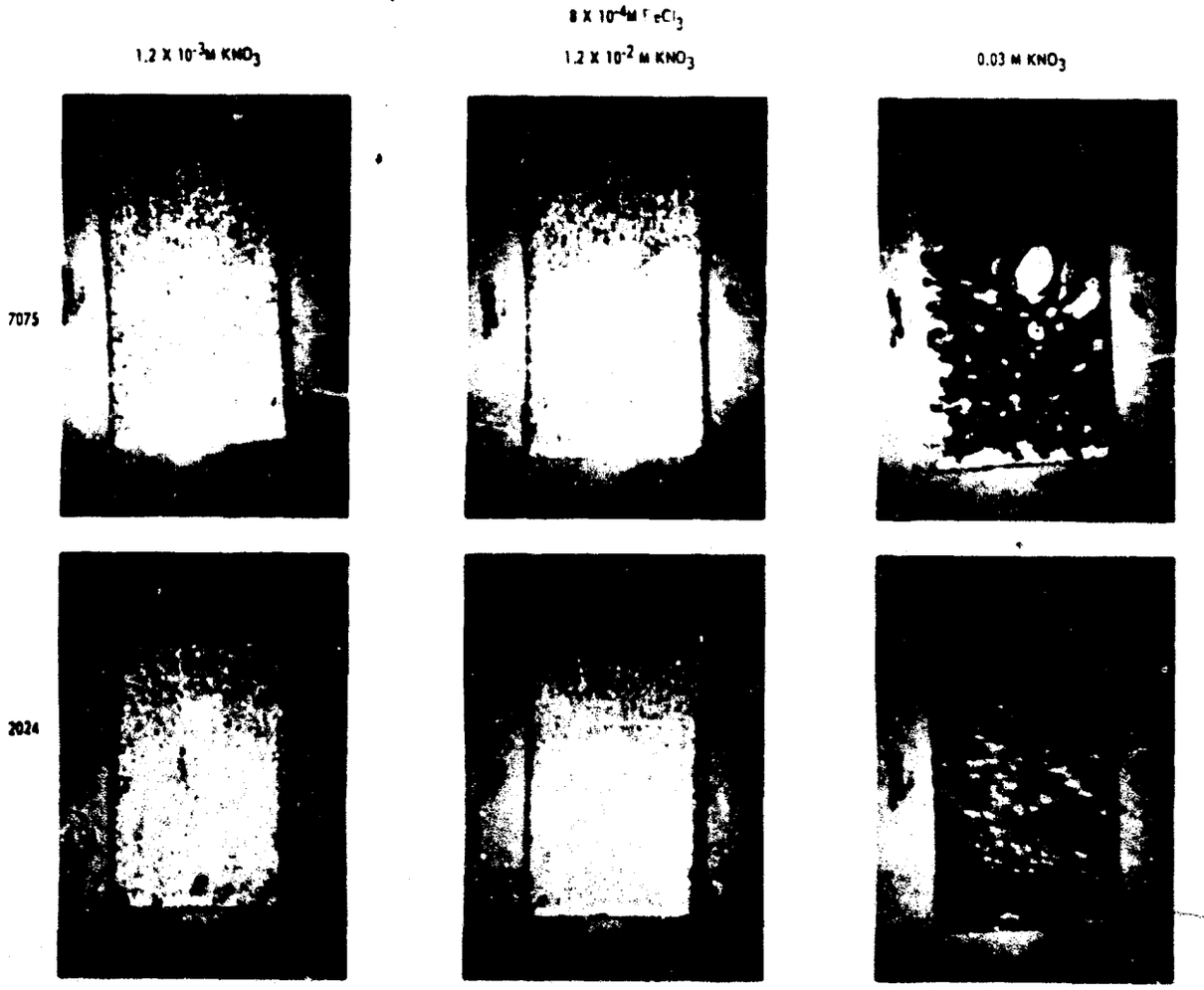
LEGEND: CONCENTRATIONS OF $FeCl_3$ FROM LEFT TO RIGHT: 8 X 10^{-6}M, 8 X 10^{-5}M, AND 8 X 10^{-4}M ALUMINUM ALLOYS 7075 (TOP) AND 2024 (BOTTOM) WERE IMMERSED IN WATER METAL SOLUTIONS, pH 7.0, TEMPERATURE 30°C FOR 48 HOURS.

Figure 1. Corrosion of 2024 and 7075 Alloys by Varying Concentrations of $FeCl_3$



LEGEND: POTASSIUM NITRATE WAS ADDED TO 8 X 10^{-4} M $FeCl_3$ TO GIVE SOLUTIONS CONTAINING (FROM LEFT TO RIGHT) 1.2 X 10^{-6} M KNO_3, 1.2 X 10^{-5} M KNO_3, 1.2 X 10^{-4} M KNO_3, 1.2 X 10^{-3} M KNO_3, 1.2 X 10^{-2} M KNO_3, AND 0.03 M KNO_3. ALUMINUM ALLOYS 7075 (TOP ROW) AND 2024 (BOTTOM ROW) WERE IMMERSED IN THESE SOLUTIONS AT pH 7.0 AND 30°C FOR 48 HOURS.

Figure 2. Corrosion of 7075 and 2024 Alloys by 8 x 10^{-4} M $FeCl_3$ in Varying Concentrations of KNO_3 (Sheet 1 of 2)



LEGEND: POTASSIUM NITRATE WAS ADDED TO 8 X 10^{-4} M $FeCl_3$ TO GIVE SOLUTIONS CONTAINING (FROM LEFT TO RIGHT) 1.2 X 10^{-4} M KNO_3, 1.2 X 10^{-3} M KNO_3, 1.2 X 10^{-4}M KNO_3, 1.2 X 10^{-3} M KNO_3, 1.2 X 10^{-2} M KNO_3, AND 0.03 M KNO_3. ALUMINUM ALLOYS 7075 (TOP ROW) AND 2024 (BOTTOM ROW) WERE IMMERSED IN THESE SOLUTIONS AT pH 7.0 AND 30°C FOR 48 HOURS.

Figure 2. Corrosion of 7075 and 2024 Alloys by 8 x 10^{-4} M $FeCl_3$ in Varying Concentrations of KNO_3 (Sheet 2 of 2)

The concentrations of nitrate inhibiting 50% aluminum corrosion caused by 8×10^{-4} M $CaCl_2$ or NaCl were determined. This concentration was 0.06 g KNO_3 per liter, 5.9×10^{-4} M KNO_3. This relationship suggested that inhibition of corrosion depends on a stoichiometric proportion between the concentration of corrosion inhibitor and the compounds stimulating corrosion. The same concentration dependence was observed with phosphate as an inhibitor.

The chemical mechanisms by which nitrate acts as a passivator are unclear. It seems highly improbable that nitrate forms stable compounds with calcium or ferric ion and thereby prevents their participation in the corrosion process. It seems equally improbable that nitrate complexes with the aluminum surface and renders it resistant to the action of corrosive ions, but the ability of nitrate to inhibit corrosion appears to depend on its concentration relative to other elements of the growth medium. It was concluded that microbial activities which remove nitrate from a medium more rapidly than iron or calcium are removed tend to make the medium more corrosive for aluminum.

3. Nitrate Utilization by Organisms Oxidizing Jet Fuel

A knowledge of certain features of microbial physiology suggested a biological mechanism by which the growth of organisms could cause aluminum corrosion. Microorganisms that grow on mineral media, such as those which oxidize fuel, utilize the various components of mineral medium at different rates and to different extents. The quantity of iron or calcium used for growth is far less than the quantity of nitrate used in the formation of cell protein and nucleic acids. It appeared, therefore, that the growth of microorganisms in media low in nitrate and phosphate would result in the removal of these radicals while the calcium, iron, and chloride present would remain in sufficient concentration to cause aluminum corrosion. Accordingly, tests were performed to determine whether organisms would remove nitrate from the growth medium and allow the corrosive ions to act on aluminum. The data in Table 1 showed that these fuel isolates could grow in the absence of ammonium ions and utilize nitrate as a sole source of nitrogen. This table shows the diminution of nitrate concentration as a function of time. In the work to be described, this fall in nitrate concentration is related to the onset of corrosion in two ways; first, corrosion occurs more rapidly in growing cultures containing small concentrations of nitrate than in uninoculated controls, and second, corrosion occurs in media initially containing high nitrate concentrations only after long periods of time when the nitrate has been depleted by cell growth and synthesis.

4. Aluminum Corrosion in Modified Bushnell-Haas Media Made to Various Concentrations of Nitrate in the Absence and Presence of Fuel Isolates

The hypothesis was tested that microorganisms in jet fuel-water systems are capable of removing corrosion inhibitors and in this way bringing about aluminum alloy corrosion.

TABLE 1

NITRATE REDUCTION BY FUEL ISOLATES IN MEDIUM CONTAINING HIGH CONCENTRATIONS OF KNO_3

Time (Day)	Nitrate (moles/liter)	(moles/liter)
0	1200×10^{-5}	0
19	9.49×10^{-5}	207×10^{-5}
43	11.9×10^{-5}	195×10^{-5}
89	7.9×10^{-5}	207×10^{-5}

Legend: Bushnell-Haas fuel medium containing 1.2 grams KNO_3 as the only nitrogen source was inoculated with fuel-grown mixed culture which had been washed 3 times in distilled water. The culture was placed on a rotary shaker at 30°C and nitrate determinations by the method of Skujins[a] and nitrate determinations by the method of Pappenhagen and Mellon[b] were made at the indicated time intervals. Initial microscopic count of the mixed inoculum was 10^7. After 89 days the microscopic count was 10^9.

a. J. J. Skujins, "Spectrophotometric Determination of Nitrate with 4-Methylumbelliferone," Anal. Chem., 36, 240 (1964).

b. J. M. Pappenhagen with M. G. Mellon, "Ultraviolet Spectrophotometric Determination of Nitrites," Anal. Chem., 25, 341 (1953).

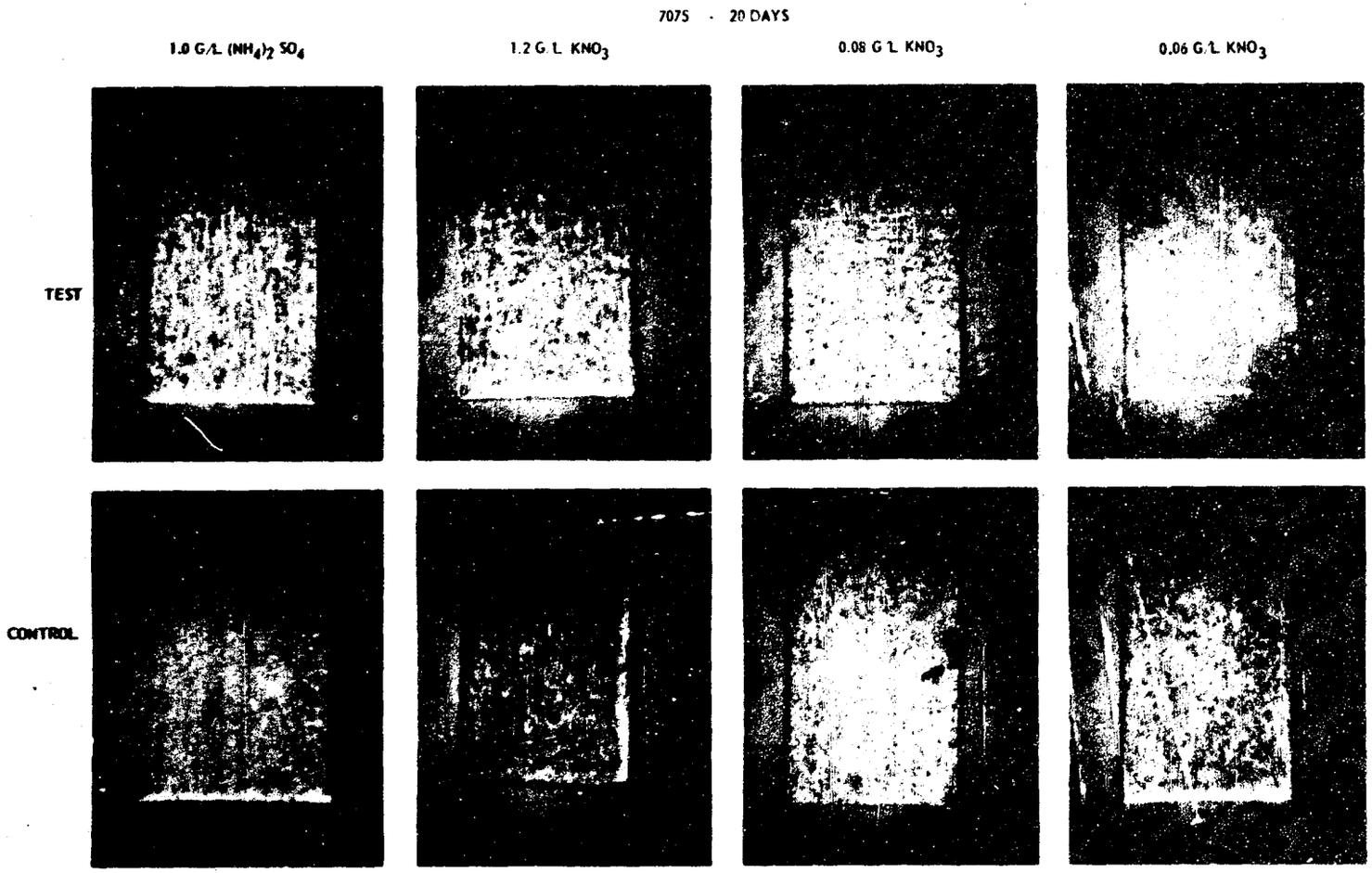
Experimental evidence suggesting this mechanism of microbial corrosion of aluminum took three forms. First, it was verified that the calcium and iron of the medium were corrosive to aluminum in the absence of other medium components. Second, tests were made showing that both nitrate and phosphate inhibit the corrosion caused by calcium and iron. Third, the ability of fuel isolates to remove nitrate during jet fuel oxidation was demonstrated.

With this background in mind tests were designed to determine if microorganisms growing in an intrinsically corrosive medium could make that medium actively corrosive. Accordingly, a series of media were prepared conforming to the basic formula of the Bushnell-Haas medium with the following exceptions: One medium contained no nitrogen source, five media contained various concentrations of potassium nitrate substituted for ammonium nitrate, and one medium contained ammonium sulfate substituted for ammonium nitrate. Thirty identical flasks were prepared of each medium. Half of the identical series was inoculated with a mixed culture of fuel-oxidizing organism; the other half was not inoculated, but was maintained as a control of microbial contamination and aluminum corrosion.

Nitrate also inhibited the chemical corrosion of the 2024 alloy caused by the ions of the growth medium. But the corrosion of this alloy brought about by microorganisms differed from that observed with the 7075 alloy. The microbial corrosion of the 2024 alloy appeared to take place only in the presence of large cell populations, while 7075 alloy corrosion was evident first at low nitrate and low cell concentration, and only much later at high nitrate and high cell concentration. Figure 6 shows that microbial growth in media containing 0.02 g KNO_3 per liter does not corrode the 2024 alloy in 97 days, but the 7075 alloy was corroded in this period of time at 0.02 g KNO_3 per liter.

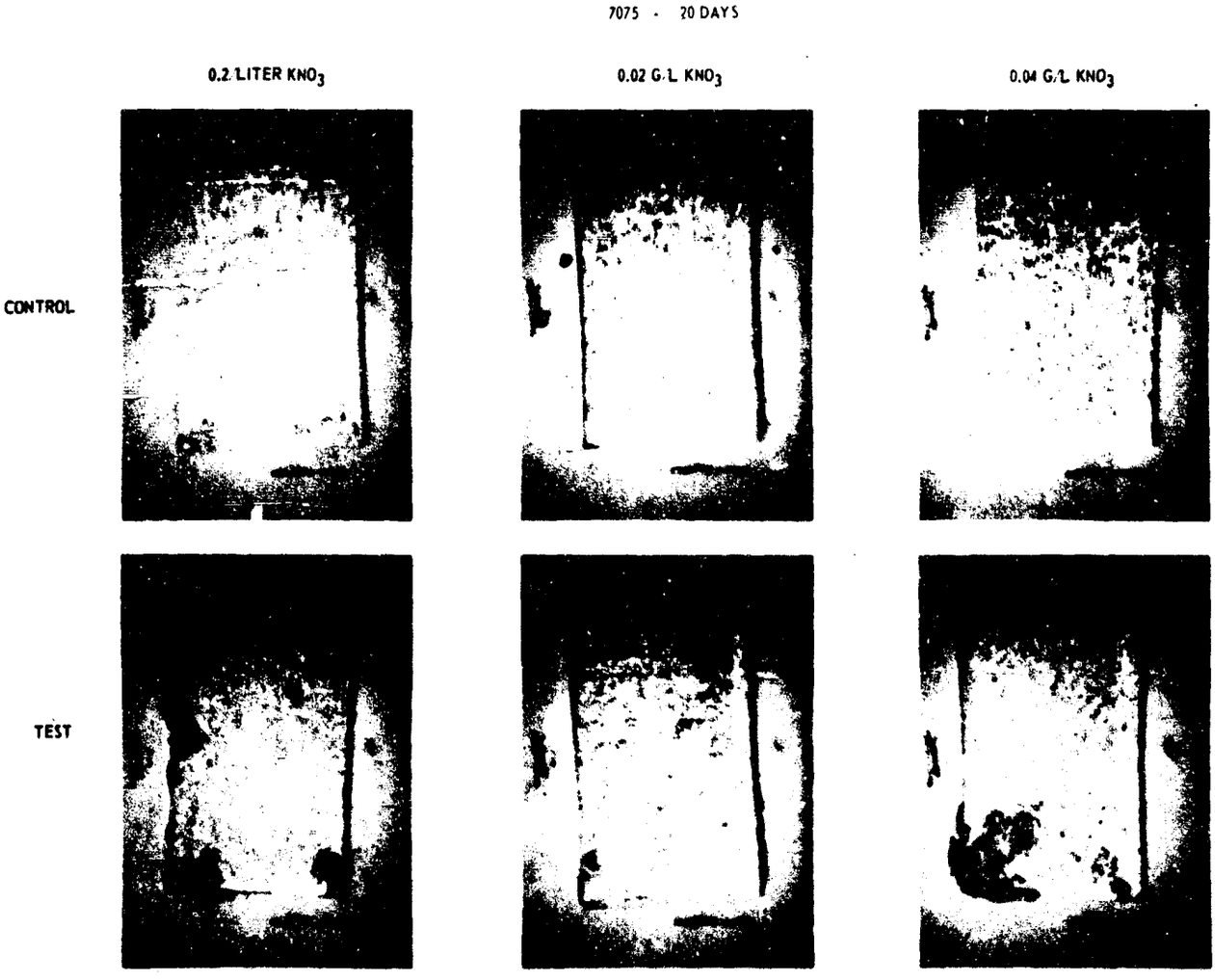
The observations recorded in Figures 3 through 6 have many implications regarding the proposed mechanism of the microbial corrosion of aluminum. The corrosion of alloy 7075 in media with little nitrate at short periods of time was anticipated. However, the absence of corrosion on 2024 in inoculated media low in nitrate, even after long periods of time, cannot be explained by the first hypothesis proposed. The occurrence of corrosion after 97 days on the two alloys could have resulted from the removal of inhibitor or nitrate; it could also have resulted from the production of corrosive organic compounds by microorganisms. As shown in a subsequent section, such organic compounds have been purified and they have been shown to stimulate aluminum corrosion.

These observations point up the ability of microbial flora to control the corrosive properties of the medium in which they grow and metabolize. These data represent a singular achievement in producing aluminum corrosion by microorganisms under rigorously controlled conditions.[5]



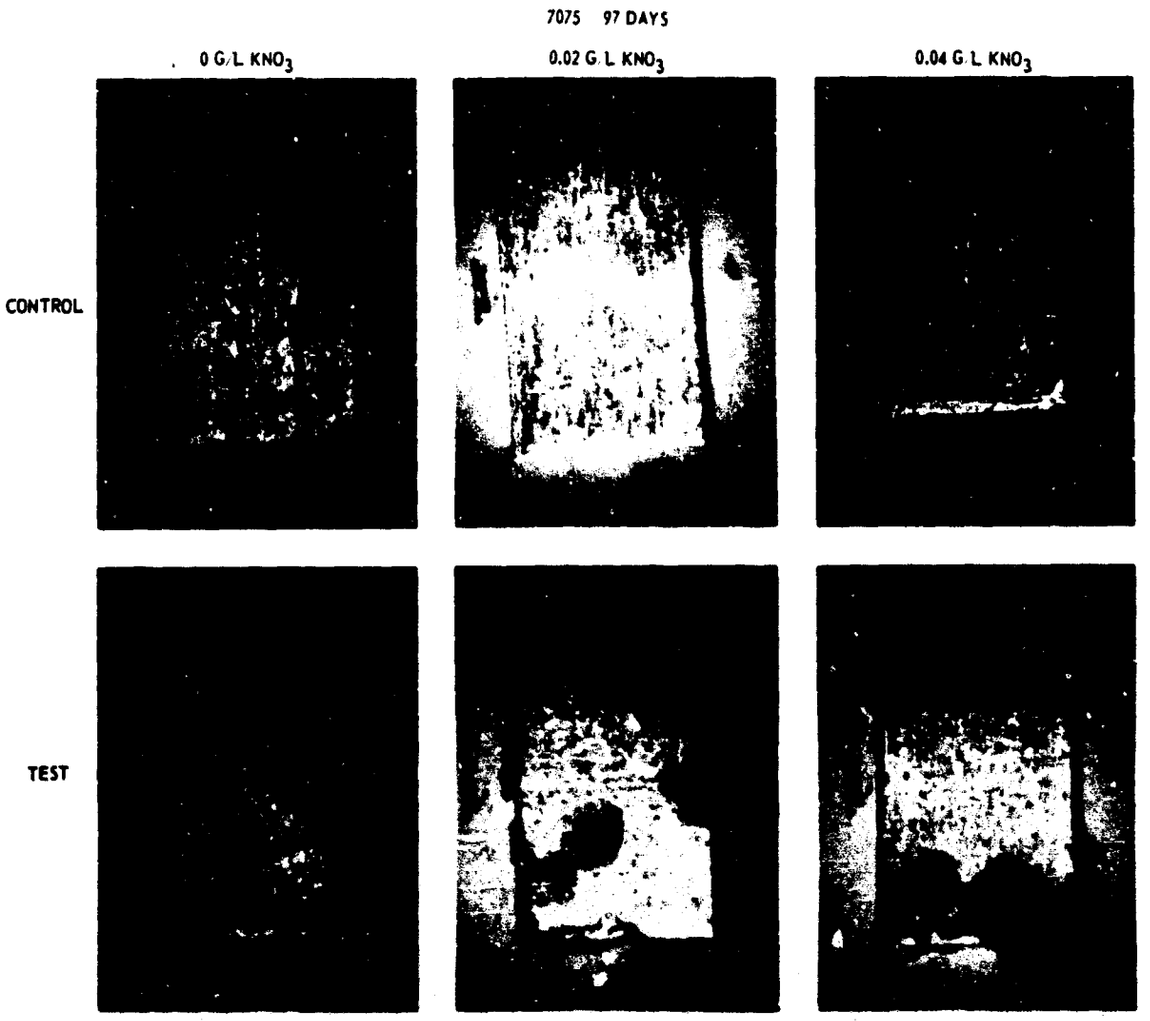
LEGEND: FLASKS WERE INOCULATED WITH 5 mL OF MIXED CULTURE WHICH HAD BEEN GROWN 48 HOURS IN NBH-FUEL, HARVESTED BY CENTRIFUGE AND WASHED 3 TIMES IN DISTILLED H_2O. AFTER INOCULATION THE FLASKS WERE PLACED AT 37°C FOR 20 DAYS ON A NEW BRUNSWICK SHAKER. THE TOP FIGURES REPRESENT UNINOCULATED CONTROL FLASKS CONTAINING SOLUTIONS, FROM LEFT TO RIGHT, OF 0 0.02, 0.04, 0.06, 0.08, AND 1.2 GMS KNO_3 PER LITER AND THE LAST SOLUTION CONTAINING 1.0 GM OF $(HN_4)_2SO_4$ PER LITER. INOCULATED TEST FLASKS ARE REPRESENTED BY THE BOTTOM ROW OF FIGURES

Figure 3. Corrosion of 7075 Alloy by Mixed Culture in Media Containing Varying Concentrations of KNO_3 as the Only Nitrogen Source (Sheet 1 of 2)



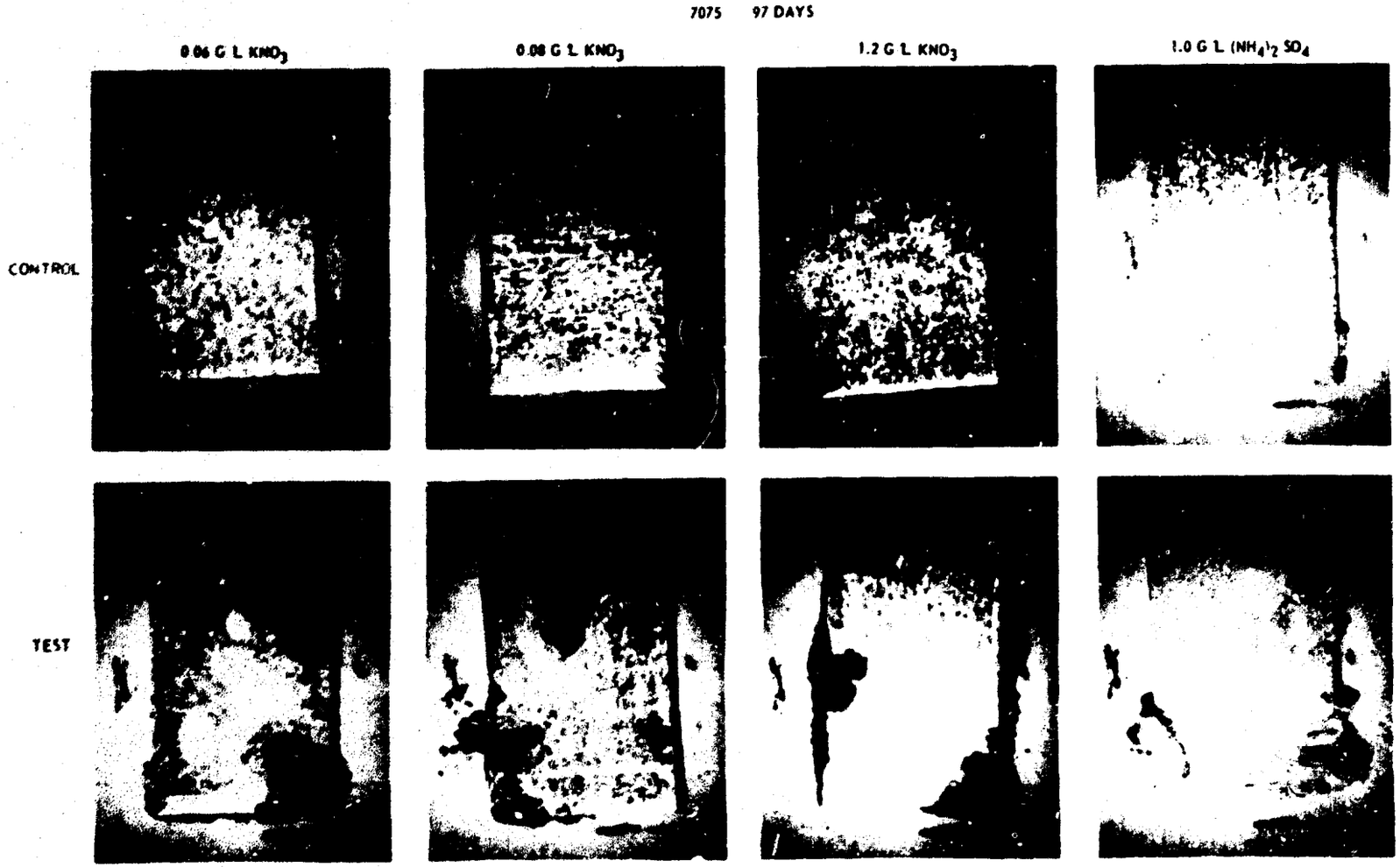
LEGEND: FLASKS WERE INOCULATED WITH 5 ML OF MIXED CULTURE WHICH HAD BEEN GROWN 48 HOURS IN NBH-FUEL HARVESTED BY CENTRIFUGE AND WASHED 3 TIMES IN DISTILLED H_2O. AFTER INOCULATION THE FLASKS WERE PLACED AT 37°C FOR 20 DAYS ON A NEW BRUNSWICK SHAKER. THE TOP FIGURES REPRESENT UNINOCULATED CONTROL FLASKS CONTAINING SOLUTIONS, FROM LEFT TO RIGHT, OF 0, 0.02, 0.04, 0.06, 0.08, AND 1.2 GMS KNO_3 PER LITER AND THE LAST SOLUTION CONTAINING 1.0 GM OF $(NH_4)_2SO_4$ PER LITER. INOCULATED TEST FLASKS ARE REPRESENTED BY THE BOTTOM ROW OF FIGURES.

Figure 3. Corrosion of 7075 Alloy by Mixed Culture in Media Containing Varying Concentrations of KNO_3 as the Only Nitrogen Source (Sheet 2 of 2)



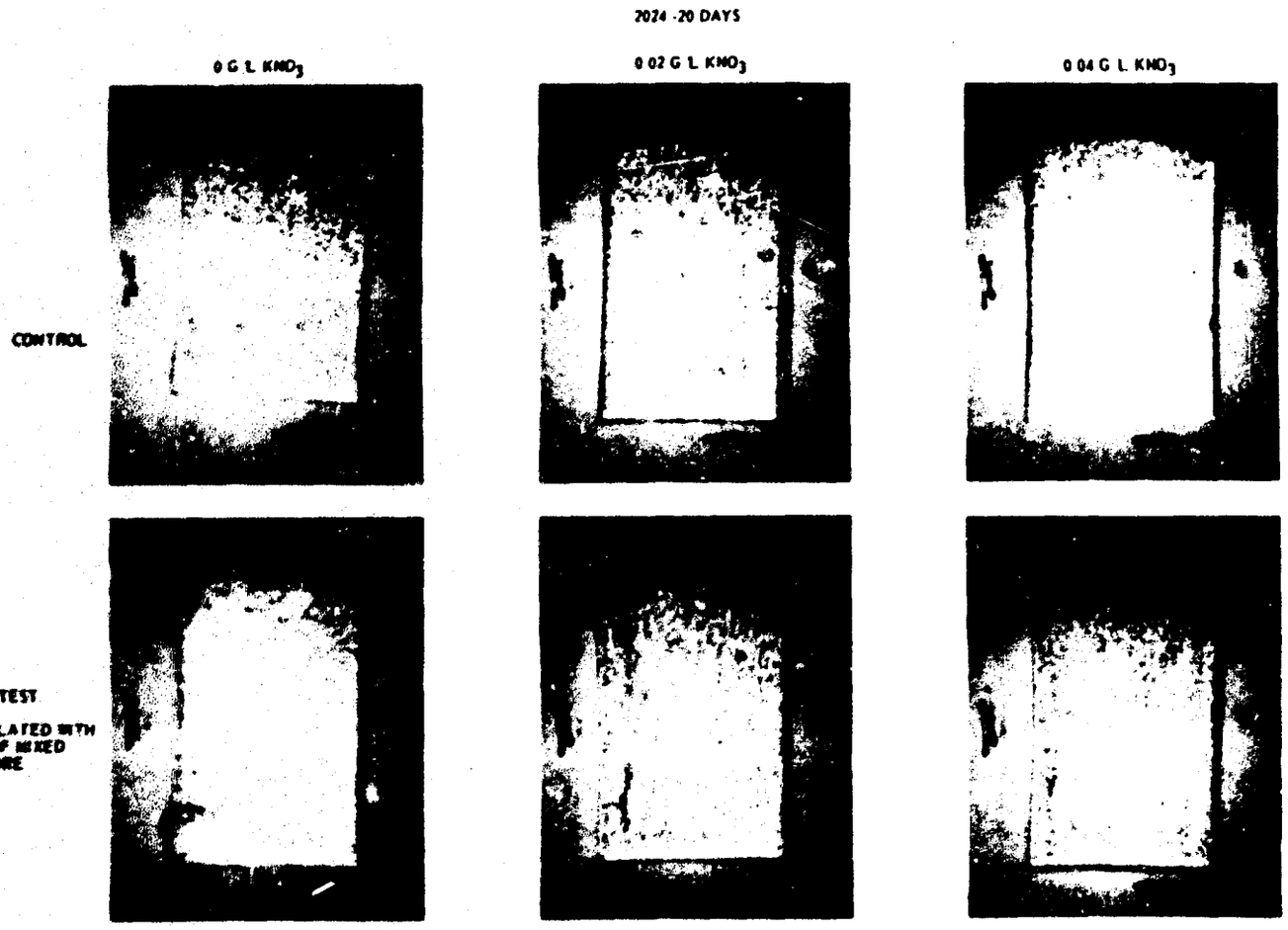
LEGEND: FLASKS WERE INOCULATED WITH 5 ML OF MIXED CULTURE WHICH HAD BEEN GROWN 48 HOURS IN NBH-FUEL, HARVESTED BY CENTRIFUGATION AND WASHED 3 TIMES IN DISTILLED H_2O. AFTER INOCULATION THE FLASKS WERE PLACED AT 37°C FOR 97 DAYS ON A NEW BRUNSWICK SHAKER. THE TOP FIGURES REPRESENT UNINOCULATED CONTROL FLASKS CONTAINING SOLUTIONS, FROM LEFT TO RIGHT, OF 0, 0.02, 0.04, 0.06, 0.08, AND 1.2 GMS KNO_3 PER LITER AND THE LAST SOLUTION CONTAINING 1.0 GM OF $(NH_4)_2SO_4$ PER LITER. INOCULATED TEST FLASKS ARE REPRESENTED BY THE BOTTOM ROW OF FIGURES.

Figure 4. Corrosion of 7075 Alloy by Mixed Culture in Media Containing Varying Concentrations of KNO_3 as the Only Nitrogen Source (Sheet 1 of 2)



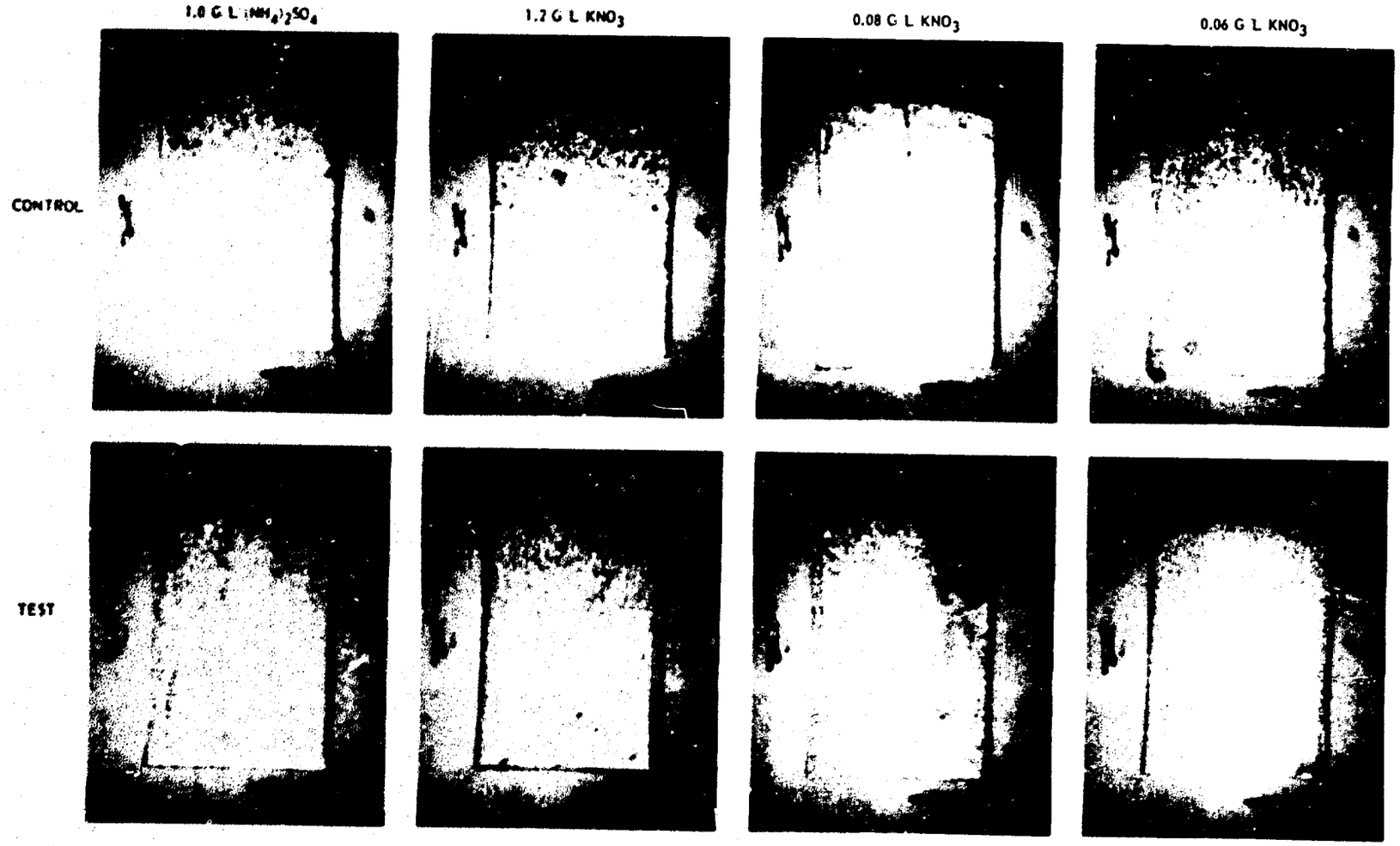
LEGEND: FLASKS WERE INOCULATED WITH 5 ML OF MIXED CULTURE WHICH HAD BEEN GROWN 48 HOURS IN NBH-FUEL, HARVESTED BY CENTRIFUGATION AND WASHED 3 TIMES IN DISTILLED H_2O. AFTER INOCULATION THE FLASKS WERE PLACED AT 37°C FOR 97 DAYS ON A NEW BRUNSWICK SHAKER. THE TOP FIGURES REPRESENT UNINOCULATED CONTROL FLASKS CONTAINING SOLUTIONS, FROM LEFT TO RIGHT OF 0, 0.02, 0.04, 0.06, 0.08, AND 1.2 GMS KNO_3 PER LITER AND THE LAST SOLUTION CONTAINING 1.0 GM OF $(NH_4)_2SO_4$ PER LITER. INOCULATED TEST FLASKS ARE REPRESENTED BY THE BOTTOM ROW OF FIGURES.

Figure 4. Corrosion of 7075 Alloy by Mixed Culture in Media Containing Varying Concentrations of KNO_3 as the Only Nitrogen Source (Sheet 2 of 2)



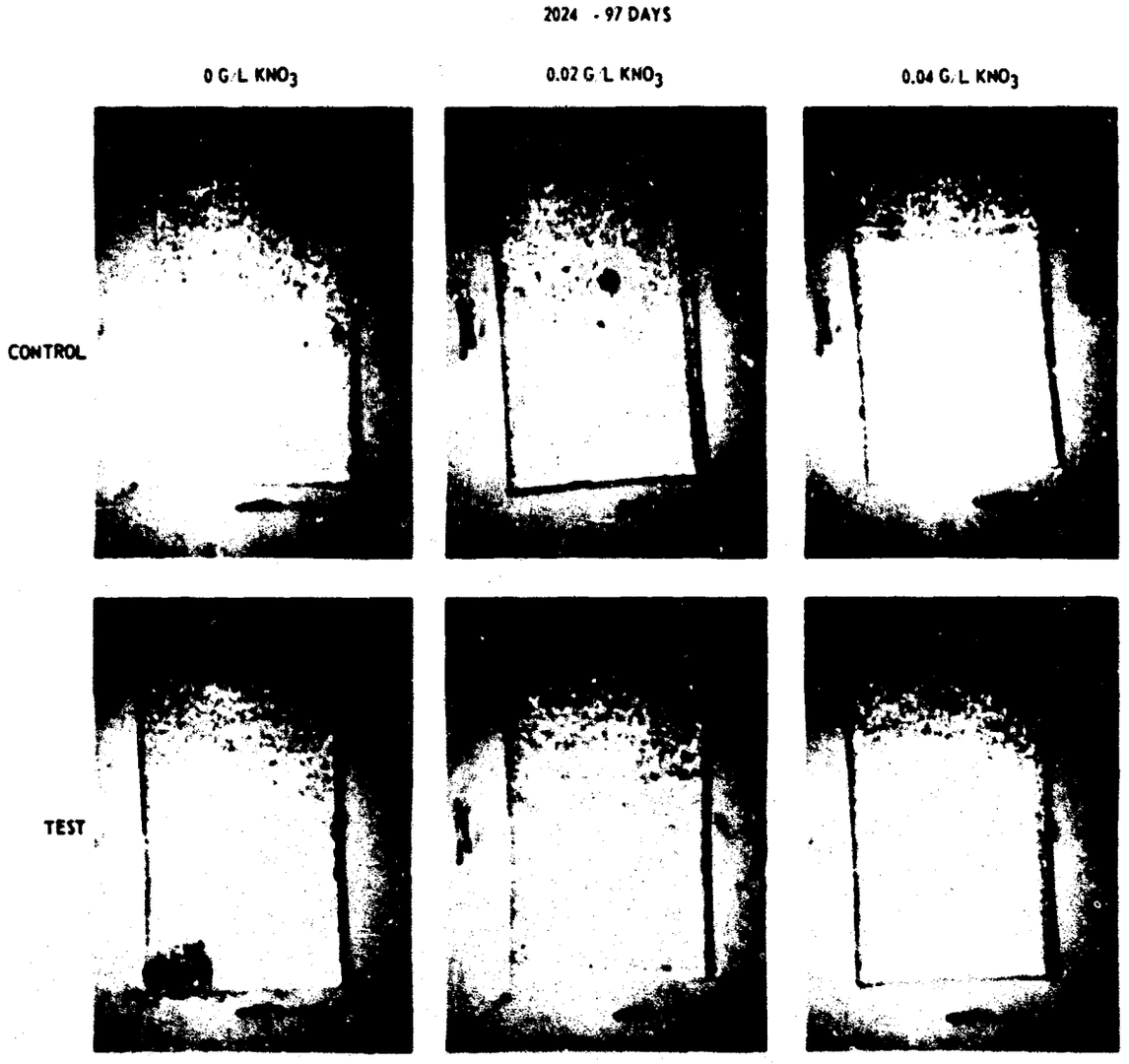
LEGEND: FLASKS WERE INOCULATED WITH 5 ML OF MIXED CULTURE WHICH HAD BEEN GROWN 48 HOURS IN MBH-FUEL, HARVESTED BY CENTRIFUGATION AND WASHED 3 TIMES IN DISTILLED H_2O. AFTER INOCULATION THE FLASKS WERE PLACED AT 37°C FOR 20 DAYS ON A NEW BRUNSWICK SHAKER. THE TOP FIGURES REPRESENT UNINOCULATED CONTROL FLASKS CONTAINING SOLUTIONS, FROM LEFT TO RIGHT, OF 0, 0.02, 0.04, 0.06, 0.08, AND 1.2 GMS KNO_3 PER LITER AND THE LAST SOLUTION CONTAINING 1.8 GM OF $(NH_4)_2SO_4$ PER LITER. INOCULATED TEST FLASKS ARE REPRESENTED BY THE BOTTOM ROW OF FIGURES.

Figure 5. Corrosion of 2024 Alloy by Mixed Culture in Media Containing Varying Concentrations of KNO_3 as the Only Nitrogen Source (Sheet 1 of 2)



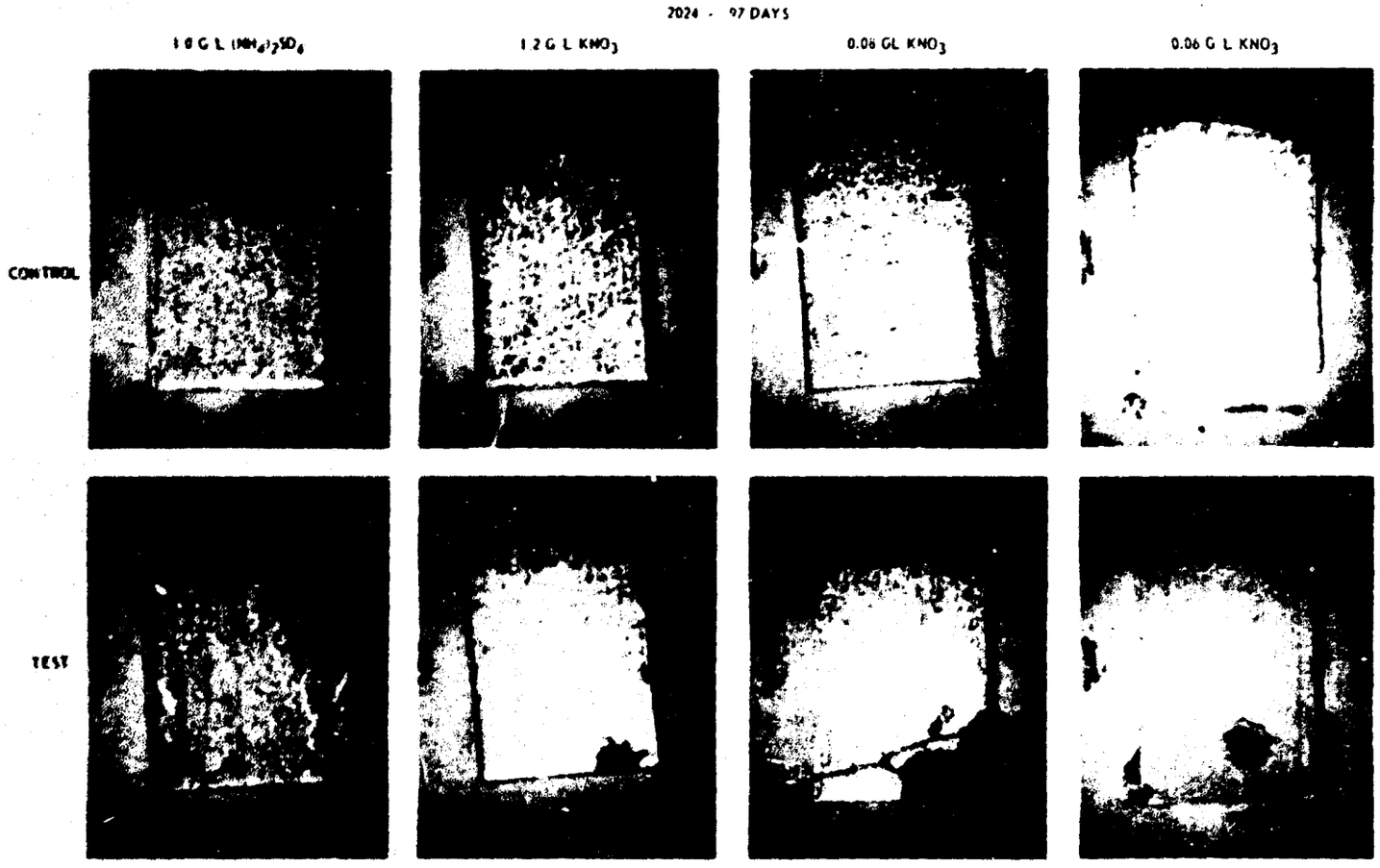
LEGEND: FLASKS WERE INOCULATED WITH 5 ML OF MIXED CULTURE WHICH HAD BEEN GROWN 48 HOURS IN NBH- FUEL, HARVESTED BY CENTRIFUGATION AND WASHED 3 TIMES IN DISTILLED H_2O. AFTER INOCULATION THE FLASKS WERE PLACED AT 37°C FOR 28 DAYS ON A NEW BRUNSWICK SHAKER. THE TOP FIGURES REPRESENT UNINOCULATED CONTROL FLASKS CONTAINING SOLUTIONS, FROM LEFT TO RIGHT, OF 0, 0.02, 0.04, 0.06, 0.08, AND 1.2 GMS KNO_3 PER LITER AND THE LAST SOLUTION CONTAINING 1.0 GM OF $(NH_4)_2SO_4$ PER LITER. INOCULATED TEST FLASKS ARE REPRESENTED BY THE BOTTOM ROW OF FIGURES.

Figure 5. Corrosion of 2024 Alloy by Mixed Culture in Media Containing Varying Concentrations of KNO_3 as the Only Nitrogen Source (Sheet 2 of 2)



LEGEND: FLASKS WERE INOCULATED WITH 5 ML OF MIXED CULTURE WHICH HAD BEEN GROWN 48 HOURS IN IN NBM-FUEL, HARVESTED BY CENTRIFUGATION AND WASHED 3 TIMES IN DISTILLED H_2O. AFTER INOCULATION THE FLASKS WERE PLACED AT 37°C FOR 97 DAYS ON A NEW BRUNSWICK SHAKER. THE TOP FIGURES REPRESENT UNINOCULATED CONTROL FLASKS CONTAINING SOLUTIONS, FROM LEFT TO RIGHT, OF 0, 0.02 0.04, 0.06, 0.08, AND 1.2 GMS KNO_3 PER LITER AND THE LAST SOLUTION CONTAINING 1.0 GM OF $(NH_4)_2SO_4$ PER LITER. INOCULATED TEST FLASKS ARE REPRESENTED BY THE BOTTOM ROW OF FIGURES.

Figure 6. Corrosion of 2024 Alloy by Mixed Culture in Media Containing Varying Concentrations of KNO_3 as the Only Nitrogen Source (Sheet 1 of 2)



LEGEND FLASKS WERE INOCULATED WITH 5 ML OF MIXED CULTURE WHICH HAD BEEN GROWN 48 HOURS IN NBH-FUEL, HARVESTED BY CENTRIFUGATION AND WASHED 3 TIMES IN DISTILLED H_2O. AFTER INOCULATION THE FLASKS WERE PLACED AT 37°C FOR 97 DAYS ON A NEW BRUNSWICK SHAKER. THE TOP FIGURES REPRESENT UNINOCULATED CONTROL FLASKS CONTAINING SOLUTIONS, FROM LEFT TO RIGHT, OF 0, 0.02, 0.04, 0.06, 0.08, AND 1.2 GMS KNO_3 PER LITER AND THE LAST SOLUTION CONTAINING 1.0 GM OF $(NH_4)_2SO_4$ PER LITER. INOCULATED TEST FLASKS ARE REPRESENTED BY THE BOTTOM ROW OF FIGURES.

Figure 6. Corrosion 2024 Alloy by Mixed Culture in Media Containing Varying Concentrations of KNO_3 as the Only Nitrogen Source (Sheet 2 of 2)

5. Microbial Corrosion of Aluminum in Growth Media of Different Phosphate Concentrations

Orthophosphate serves a dual function in the growth media of bacteria. It is a source of phosphate for cell growth, and it is a weak acid which buffers the medium near neutrality. Phosphorus, like nitrogen and carbon but unlike iron, magnesium, and calcium, constitutes the macrostructures of the bacterial cell. Orthophosphate passivates aluminum as well as functioning as a macro constituent of the microbial cell. A study was therefore set up to investigate the ability of fuel-oxidizing bacteria to utilize phosphate and by so doing, make the medium in which growth takes place more corrosive toward aluminum.

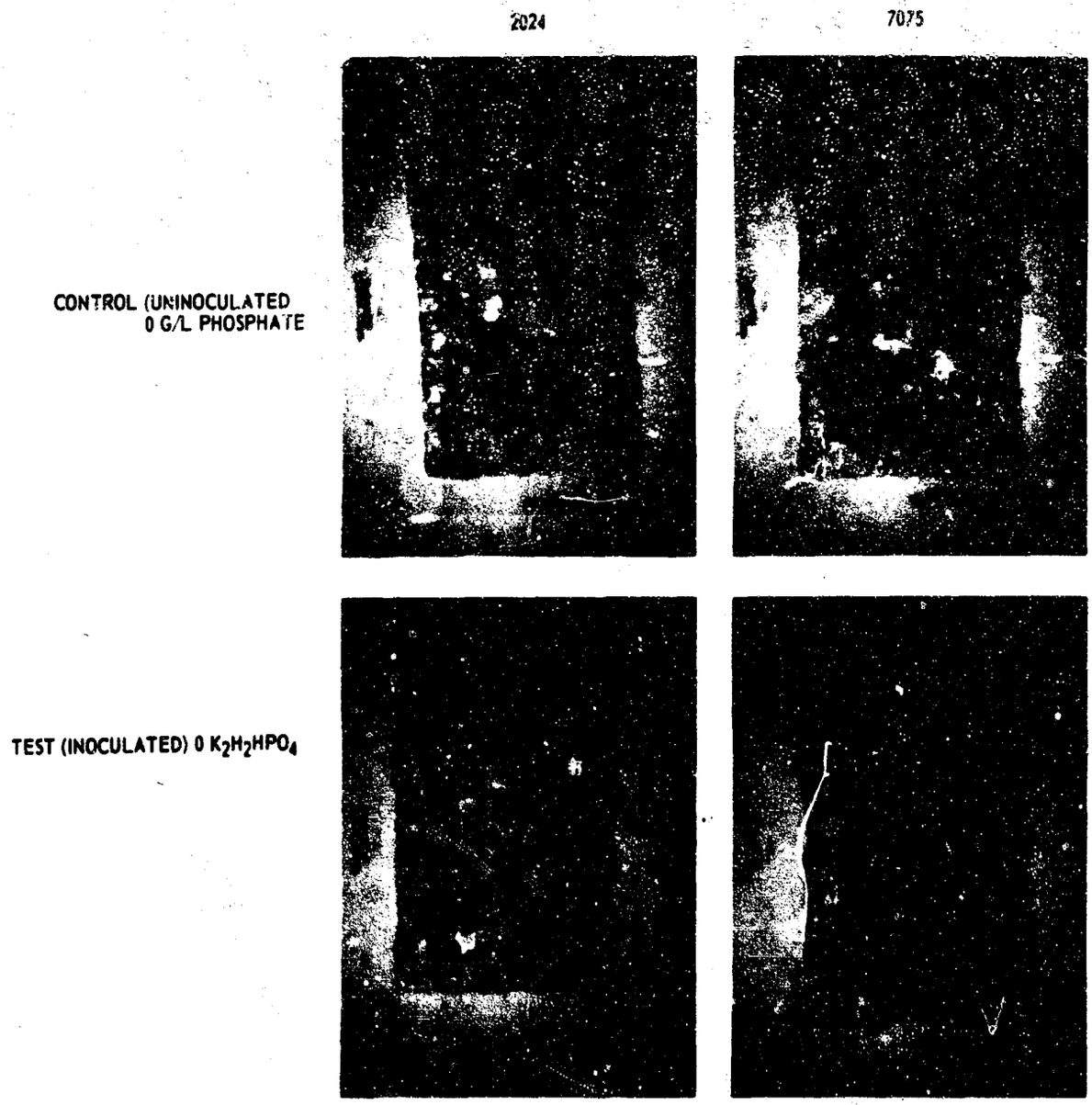
The high cell population obtained in a medium which limited nitrate and phosphate concentrations revealed the possibility of testing the ability of organisms to fix phosphate within the cell and thus enhance the corrosivity of the medium in which they grow. The medium designed in this study also permits the growth of fuel-oxidizing organisms to relatively high population densities, while at the same time the medium appears to be minimally inhibitory to corrosion. Experiments were designed to test the ability of fuel organisms to cause corrosion in these media with various low concentrations of phosphate. The basic medium contained:

0.2 g/liter .	$MgSo_4 7H_2O$
0.2 g/liter .	$CaCl_2$
0.5 g/liter	$FeCl_3$
0.06 g/liter	KNO_3

From this basic medium, four test media were prepared. The basic medium without additions, and the basic medium with 0.1, 0.3, and 0.5 g K_2HPO_4 per liter added.

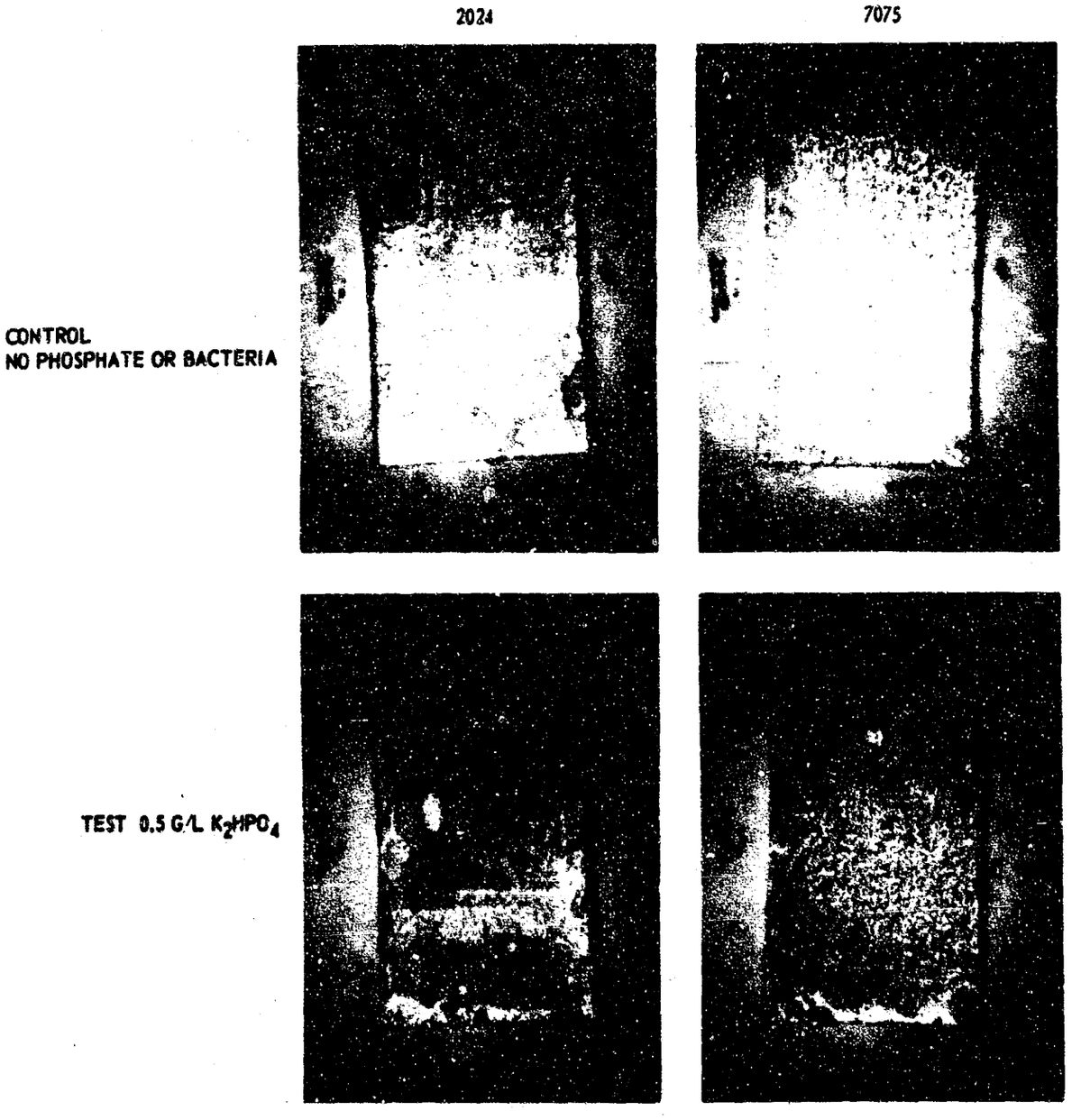
Each medium was dispensed into flasks and overlaid with JP-4 fuel. The corrosivity of the sterile medium was determined by adding alloy bars to each medium. Observations were made at 24 and 72 hours. Most corrosion occurred in media without phosphate (Figure 7) and the least corrosion occurred in media with 0.5 g K_2HPO_4 per liter (Figure 8).

Sterile media with aluminum coupons were inoculated with cells of the fuel isolate, culture 96. After 7 days incubation, no difference in corrosion was observed in the inoculated and control media without phosphate added. Figure 7 shows that in the absence of phosphate, this medium causes corrosion to about the same extent in the presence as in the absence of microorganisms. Similar results were obtained with media containing 0.3 g/liter of K_2HPO_4, but after 7 days growth, corrosion was seen in inoculated media containing 0.5 g/liter of K_2HPO_4; however, corrosion was essentially absent from aluminum coupons in the uninoculated control, Figure 8.



LEGEND: FROM LEFT TO RIGHT, 2024 AND 7075 ALLOYS ARE REPRESENTED. CONTROL STRIPS PRESENTED AT THE TOP WERE IMMERSED IN MEDIA CONTAINING NO PHOSPHATE AND NO BACTERIA. THE BOTTOM FIGURES REPRESENT TEST STRIPS WHICH WERE IMMERSED IN MEDIA OF THE SAME COMPOSITION, INOCULATED WITH 5 MLS OF BACTERIAL CULTURE. THE FIGURES REPRESENT THE ALLOYS AFTER 7 DAYS INCUBATION AT 30°C.

Figure 7. The Corrosion of Aluminum Alloys in Culture 96 in Medium Containing no Phosphate



LEGEND: FROM LEFT TO RIGHT, 2024 AND 7075 ALLOYS ARE REPRESENTED. CONTROL STRIPS PRESENTED AT THE TOP WERE IMMERSED IN MEDIA CONTAINING NO PHOSPHATE AND NO BACTERIA. THE BOTTOM FIGURES REPRESENT TEST STRIPS WHICH WERE IMMERSED IN MEDIA OF THE SAME COMPOSITION, INOCULATED WITH 5 MLS OF BACTERIAL CULTURE. THE FIGURES REPRESENT THE ALLOYS AFTER 7 DAYS INCUBATION AT 30°C.

Figure 8. The Corrosion of Aluminum Alloys in Culture 96 in Media Containing 0.5 Grams of Phosphate per Liter

The chemical mechanism responsible for phosphate inhibition of aluminum corrosion is unclear, but the formation of complex compounds of aluminum and phosphate, or phosphate and some unknown ion catalizing corrosion is a possibility. The inhibition of corrosion by nitrate, however, is probably not accomplished by this mechanism. Both nitrate and phosphate appear to be taken up by the bacteria cell and this activity has been shown to be accompanied by increases in the corrosivity of the growth medium.

6. Aluminum Corrosion in Proteinacious Media

Jet fuel water bottoms contain microorganisms which do not grow on mineral media with hydrocarbon overlays. This observation made in the ecology study suggests that such water bottoms contain a variety of contaminants which may be used as sources of carbon.

The presence of organisms in fuel-water bottoms, which are incapable of oxidizing fuel, suggests that these environments contain a variety of organic contaminants that may be used as sources of carbon.

These carbon sources may derive from lysed organisms, from organic matter, from soil run-off, or from material taken into aircraft breathers. This material may cause aluminum corrosion directly or may stimulate the production of corrosive compounds by organisms present in water bottoms which do oxidize fuel.

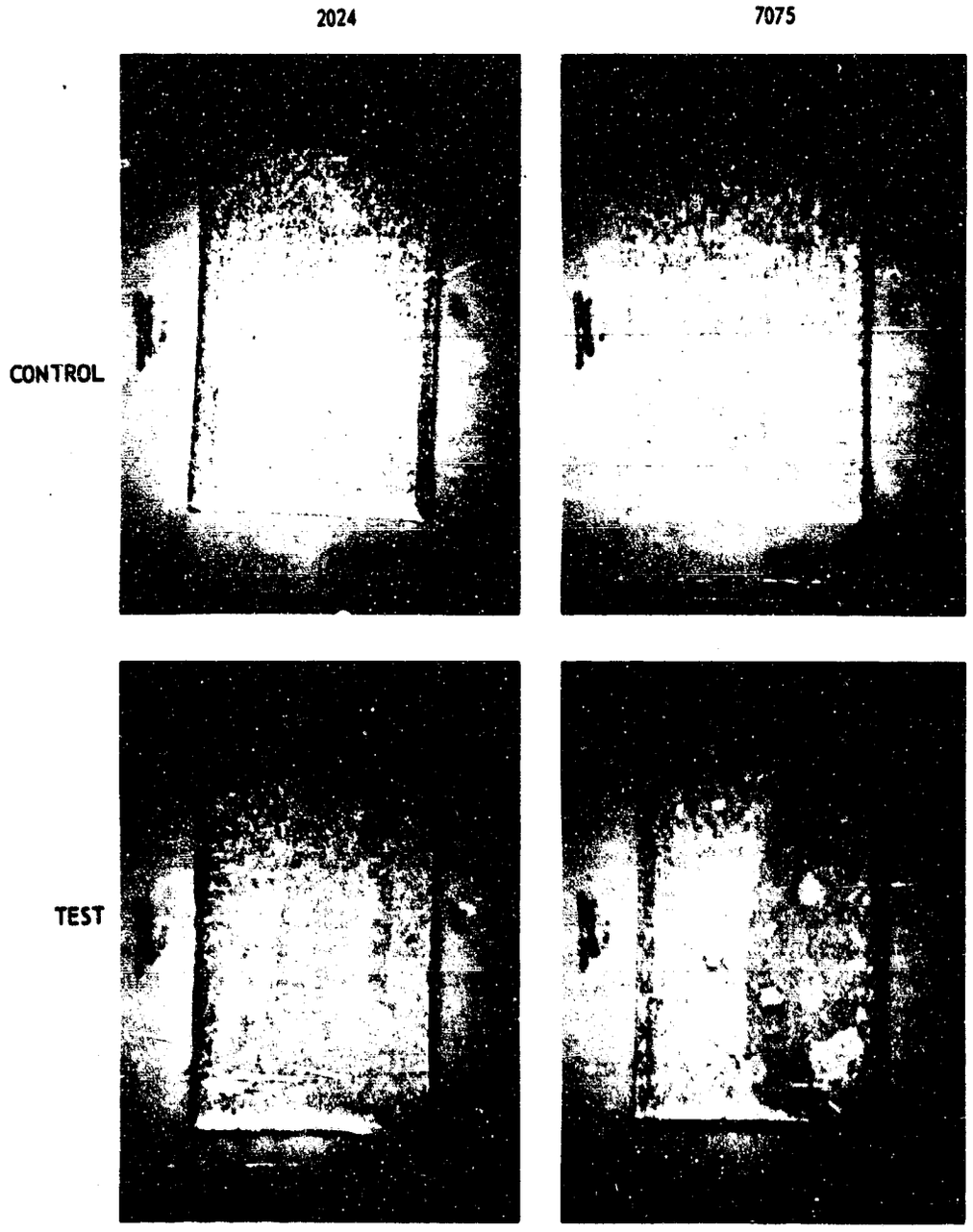
To test the effects of different media containing proteinacious material on the ability of fuel isolates to cause corrosion, three media were employed.

A 5% solution of casein was prepared in distilled H_2O and pH was adjusted to 7.0. This solution was dispensed into Erlenmeyer flasks. The casein hydrolysate at 1.2×10^{-2} M KNO_3, and casein hydrolysate plus Bushnell-Haas salts were prepared.

All media were overlayed with JP-4 fuel. As an inoculum 5 ml of culture 101 containing 1.9×10^{8} organisms per ml were added to 5 test flasks of each medium. Observations were made at 4, 7, and 15 days.

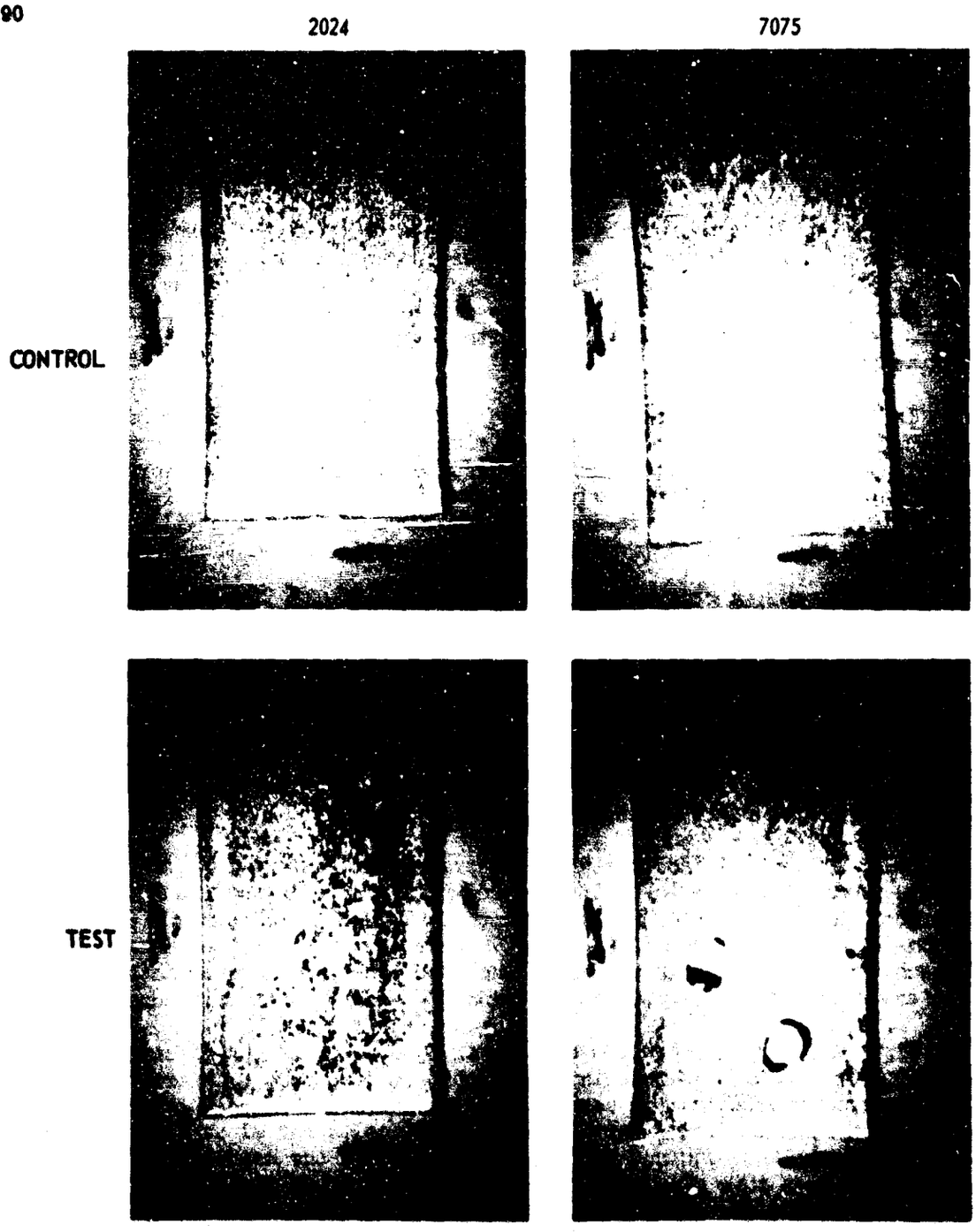
As shown in Figure 9, corrosion is produced by microorganisms in casein alone on the 2024 and 7075 alloy. As observed previously, the 7075 alloy is appreciably more affected by microbial corrosion than 2024.

The effect of nitrate on the corrosion of aluminum alloys by bacteria grown in casein hydrolysis is shown in Figure 10. The microbial corrosion is more severe in the presence of nitrate than in the absence of this compound. These results suggest that the corrosion produced by growth in casein hydrolysate was produced by a mechanism differing from the operative in an inorganic medium with a fuel overlay.



LEGEND: CONTROL BARS OF 2024 AND 7075 ALLOY ARE REPRESENTED BY THE TOP FIGURES. TEST FIGURES ARE PRESENTED AT THE BOTTOM; THEY WERE IMMERSED IN MEDIA THAT CONTAINED CULTURE 101. THE INOCULUM WAS GROWN IN FUEL HARVESTED BY CENTRIFUGATION AND WASHED 3 TIMES IN DISTILLED H_2O. TIME OF TEST--15 DAYS.

Figure 9. Corrosion of Aluminum Alloys in 5% Casein Hydrolysate Medium Containing Culture 101



LEGEND: UNINOCULATED CONTROLS ARE PRESENTED AT THE TOP. THE 2024 IS PRESENTED AT THE LEFT AND THE 7075 AT THE RIGHT. TEST ALLOYS REPRESENTED AT THE BOTTOM WERE IMMERSED IN MEDIA CONTAINING FUEL-GROWN CELLS WHICH HAD BEEN WASHED IN DISTILLED WATER 3 TIMES. TIME OF TEST--15 DAYS.

Figure 10. The Effect of Nitrate on the Corrosion of Aluminum Alloys by Culture 101 in 5% Casein Hydrolysate

It should be emphasized that the concentration of nitrate used in this casein medium was sufficient to prevent the occurrence of corrosion in 97 days in a sterile medium containing potentially corrosive ions.

The effect of casein hydrolysate plus the salts of the Bushnell-Haas medium is shown in Figure 11. The greatest corrosion was observed in this medium. The extent of this corrosion suggests that some property or constituent of casein destroys the ability of the nitrate to inhibit corrosion caused both by growth on casein and by the corrosive cations of the Bushnell-Haas medium.

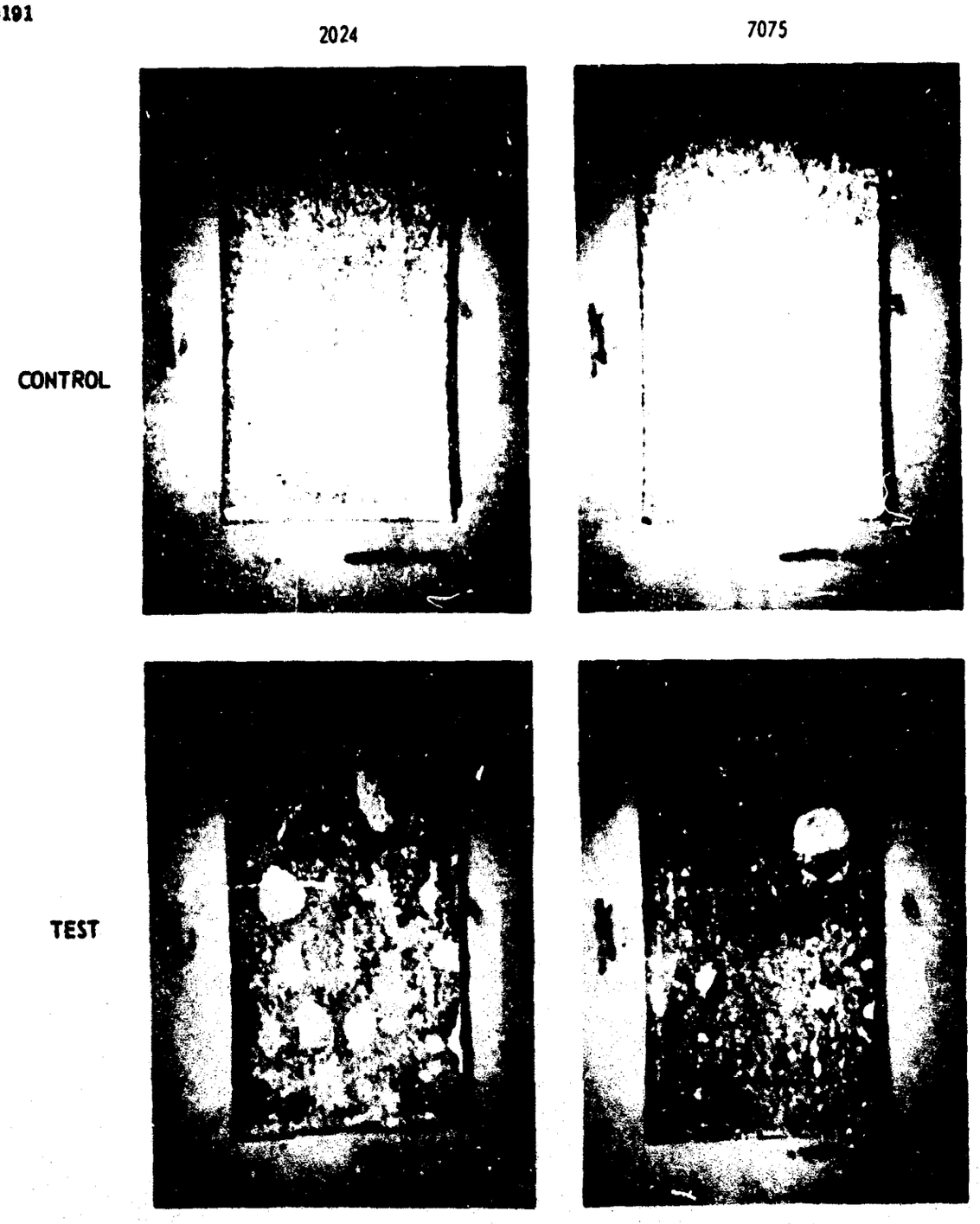
In the past, the theory was tested to determine whether microbial corrosion of aluminum takes place because microorganisms utilize naturally occuring corrosion inhibitors. These observations with casein hydrolysate suggest a second mechanism of microbial corrosion in which the organisms actually produce materials that cause corrosion. Future work will be concerned with the effect of products from lysed organisms on the corrosion of aluminum.

7. Studies of Direct Aluminum Alloy Oxidation by Metabolically Coupled Electron Mediators

The hypothesis was investigated that microorganisms produce substances acting as mediators in electron transfer from metal to oxygen or from metal to cell to oxygen. In this study methylene blue was used as a model electron transfer mediator. This dye can react both with the electron transport system of the cell and with molecular oxygen.

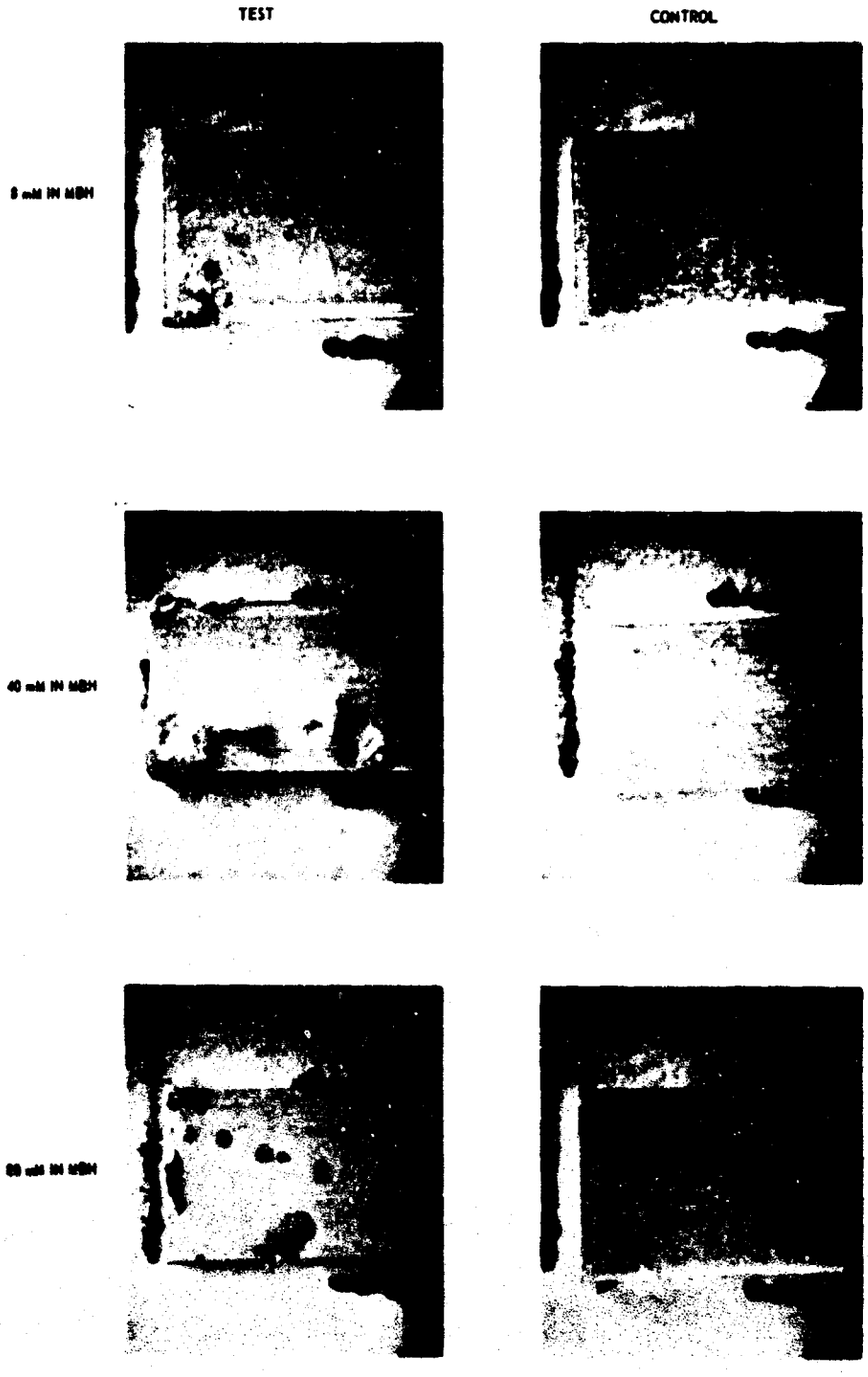
Cultures of fuel isolates were prepared in NEH with a JP-4 fuel overlay. Culture media were made to 8, 40, and 80 millimolar in methylene blue hydrochloride. The media were inoculated with fuel isolate culture 101, and coupons of aluminum alloys 2024 and 7075 were submerged in the aqueous phase of each culture. Controls contained the same concentrations of methylene blue and aluminum coupons but they were not inoculated with microorganisms.

Figures 12 and 13 permit a comparison to be made of the effect of methylene blue on aluminum coupons in the presence and absence of microbial growth. The pictures were taken five days after inoculation. The cell concentration changed from 5×10^8 cells per ml to 3.2×10^9 cells per ml during this time. The dye affects the aluminum surface very little in the absence of microbial growth, but when this electron mediator and microorganisms are included in the same culture, the organisms adhere to the surface of the aluminum. It was observed that pitting corrosion had occurred beneath the adsorbed organisms or debris. It is believed that the effect of methylene blue was predominantly on the metabolic activity of the microorganisms rather than on the surface of the aluminum coupon. These results emphasize again the necessity of carefully evaluating the environment and medium in which microorganisms cause corrosion.



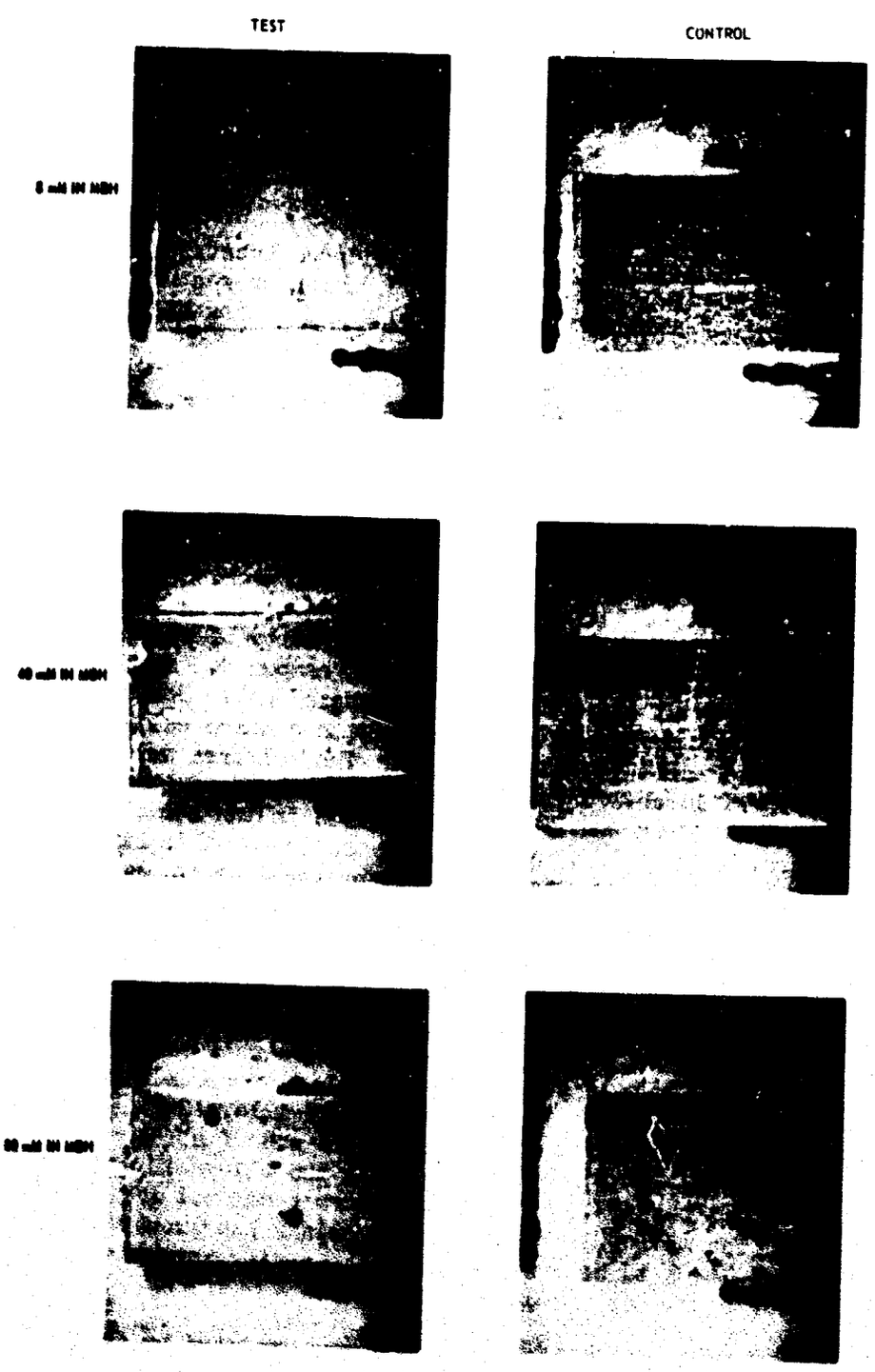
LEGEND: UNINOCULATED CONTROLS ARE PRESENTED AT THE TOP. THE 2024 IS PRESENTED AT THE LEFT AND THE 7075 AT THE RIGHT. TEST ALLOYS REPRESENTED AT THE BOTTOM WERE IMMERSED IN MEDIUM CONTAINING FUEL-GROWN CELLS WHICH HAD BEEN WASHED IN DISTILLED WATER 3 TIMES. TIME OF TEST--15 DAYS.

Figure 11. The Effect of BH-salts on the Corrosion of Aluminum Alloys by Culture 101 in 5% Casein Hydrolysate



LEGEND. THE MBH CULTURE MEDIUM WAS MADE 0, 40, AND 80 mM IN MB. THE FLASKS WERE INOCULATED WITH CULTURE 181 AND ALLOY 7075 WAS ADDED TO EACH FLASK. CONTROL FLASKS PREPARED SIMULTANEOUSLY CONTAINED NO MICROORGANISMS. TESTS AND CONTROLS WERE INCUBATED 5 DAYS AT 30°C. CONTROLS ARE PRESENTED ON THE RIGHT, TESTS ON THE LEFT. FROM TOP TO BOTTOM MB CONCENTRATION IS 0, 40, AND 80 mM RESPECTIVELY.

Figure 12. A Comparison of the Effect of Methylene Blue Hydrochloride (MBH) on Aluminum Alloy 7075 in the Presence of Microbial Growth



LEGEND: THE NBN CULTURE MEDIUM WAS UNDER 8, 40, AND 80 mM IN MB. THE FLASKS WERE INOCULATED WITH CULTURE 161 AND ALLOY 2024 WAS ADDED TO EACH FLASK. CONTROL FLASKS PREPARED SIMULTANEOUSLY CONTAINED NO MICROORGANISMS. TESTS AND CONTROLS WERE INCUBATED 3 DAYS AT 30°C. CONTROLS WERE PRESENTED ON THE RIGHT, TESTS ON THE LEFT. FROM TOP TO BOTTOM MB CONCENTRATION IS 8, 40, AND 80 mM RESPECTIVELY.

Figure 13. A Comparison of the Effect of Methylene Blue Hydrochloride (MBH) on Aluminum Alloy 2024 in the Presence of Microbial Growth

8. The Production of Corrosive Compounds by Fuel Isolates Oxidizing Jet Fuel

The first comparatively unambiguous evidence substantiating the hypothesis that microorganisms produce corrosive compounds was obtained indirectly with old cultures of fuel isolates initially containing high concentrations of nitrate.

It was previously shown that aluminum coupons submerged in Bushnell-Haas medium, with 1.2 g KNO_3 substituted for $(NH_4)_2SO_4$, for 97 days corroded. This corrosion was at first attributed to the diminution of nitrate in the medium and the presence of iron and calcium. With the objective of testing the corrosivity of these old cultures, fresh coupons were submerged in 97-day-old cultures initially with 1.2 g KNO_3. Corrosion was observed in 48 hours on aluminum coupons submerged in these cultures. (See Figure 14.)

It was believed that the increased corrosivity of the medium resulted from the microbial production of a corrosive compound. To test this assumption, modified Bushnell-Haas medium was prepared which contained 1.2 g KNO_3 per liter; and this medium was inoculated with a mixed culture of microorganisms isolated from jet fuel systems. The cultures were placed on a rotary shaker and incubated for 86 days. At the end of this period coupons of aluminum alloys 2024 and 7075 were immersed in the medium and inspected after 48 hours. Figure 14 shows that, by the end of this brief incubation period, both alloys were corroded severly by these media, but coupons in sterile media did not corrode. The corrosion occurring in these exhausted media was attributed to the presence of ferric hydroxide and calcium ion and to the absence of nitrate.

Other tests were run to better define the relationship of the advent of corrosion and nitrate depletion in these cultures. Accordingly 1.2 g KNO_3 was added to these exhausted cultures which had previously contained this concentration of nitrate. Aluminum coupons of alloys 2024 and 7075 were submerged in these media and inspected at the end of 48 hours. Figure 15 shows the corrosion which took place on the two alloys in media to which nitrate was added for the second time. Nitrate did not inhibit the corrosion stimulated by the medium in which microorganisms had grown for protracted time periods. It is to be observed that the medium which had not supported microbial growth was not itself corrosive.

These results suggest that compounds are produced by fuel isolates which cause corrosion independently of the corrosion which may be stimulated by the presence of cations in the medium, such as iron and calcium. The corrosive action of these compounds appears to take place in the presence of inhibitors such as nitrate and phosphate.

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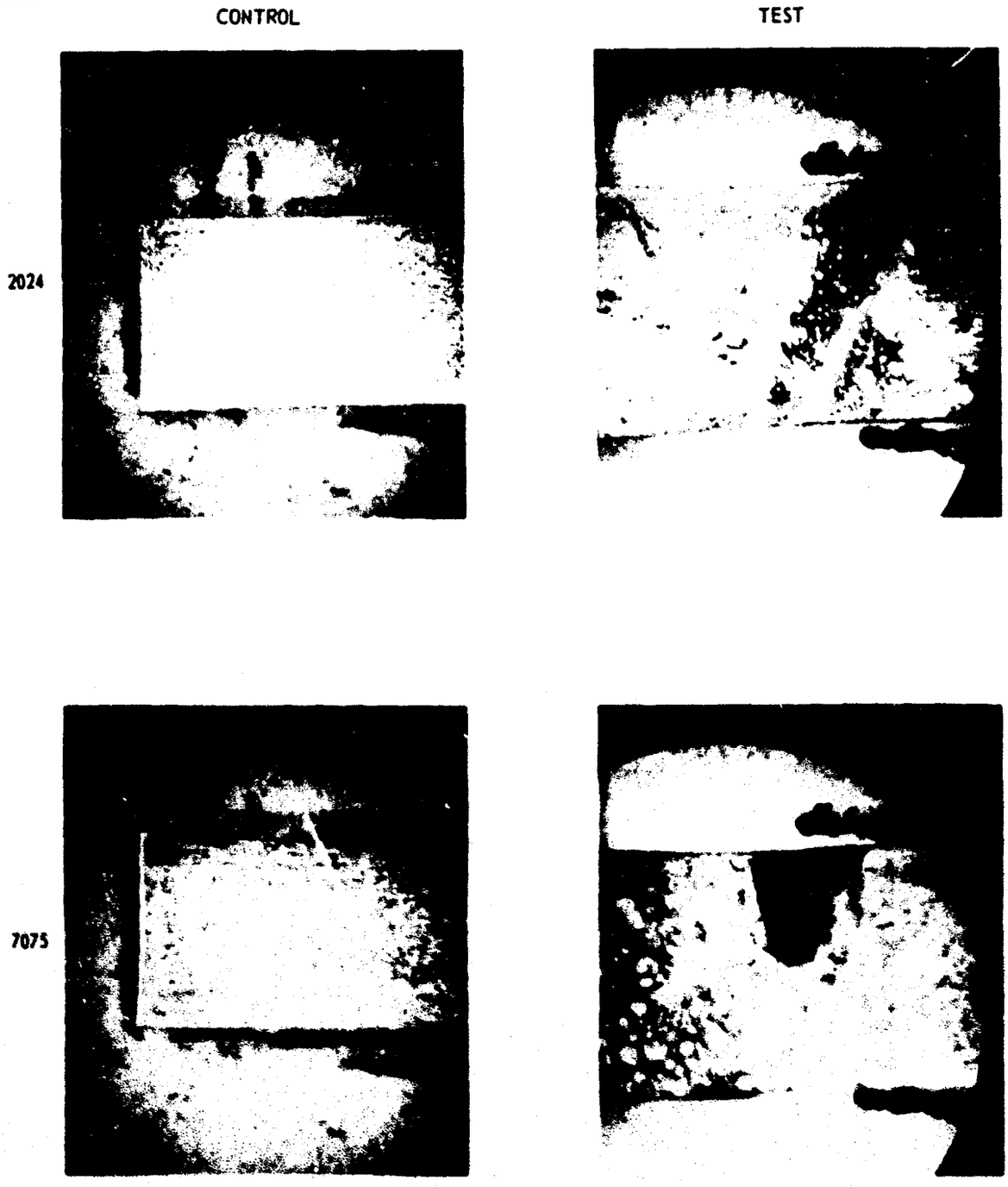
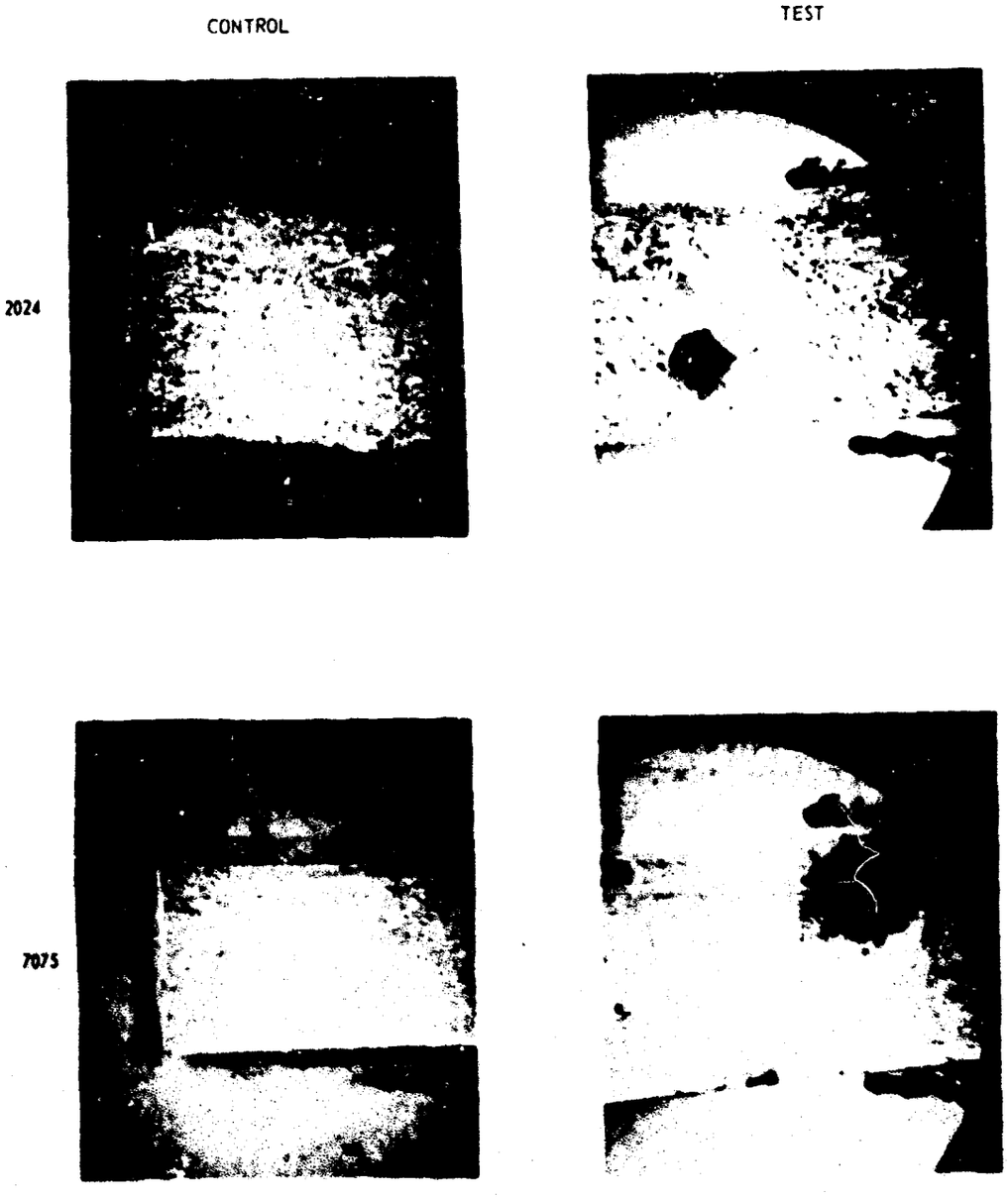


LEGEND. FLASKS OF NBH MEDIUM WITH 1.2 g KNO_3 WERE INOCULATED WITH A MIXED CULTURE OF FUEL ISOLATES AND INCUBATED AT 30°C FOR 86 DAYS. ALLOYS 7075 AND 2024 WERE THEN ADDED AND THE FLASKS WERE INCUBATED FOR 48 HOURS. CONTROL BARS OF ALLOY 2024 AND 7075 WHICH WERE NOT IMMERSED IN CORROSIVE MEDIUM ARE PRESENTED ON THE LEFT. 7075 ALLOYS ARE IN THE BOTTOM ROW AND 2024 ALLOYS IN THE TOP ROW.

Figure 14. Corrosivity of Medium Following Long Term Microbial Growth



LEGEND. FLASKS OF NBM MEDIUM WITH 1.2 gm KNO_3 WERE INOCULATED WITH A MIXED CULTURE OF FUEL ISOLATES AND INCUBATED AT 30°C FOR 86 DAYS. ALLOYS 7075 AND 2024, AND A SECOND QUANTITY OF KNO_3 (1.2 GRAMS PER LITER) WERE THEN ADDED AND THE FLASKS WERE INCUBATED FOR 48 HOURS. CONTROL BARS OF EACH ALLOY WHICH WERE NOT IMMERSED IN CORROSIVE MEDIUM ARE PRESENTED ON THE LEFT. 7075 ALLOYS ARE PRESENTED IN THE BOTTOM ROW AND 2024 ALLOYS IN THE TOP ROW.

Figure 15. The Effect of Second Addition of KNO_3 on the Corrosivity of Medium Following Long-Term Microbial Growth

B. The Chemical Fractionation of Media Made Corrosive by Microbial Growth

It was shown previously that media initially high in nitrate and which supported the growth of fuel isolates for long periods of time became corrosive. During this period such media were chemically fractionated using the anion exchange resin Dowex - 1.

The resin was in the chloride form initially and then following an HCl wash it was adjusted to pH 7 with NaCH and subsequently washed several times in water. The growth medium tested contained a mixed culture of jet fuel isolates. The cultural medium was characteristically brown after many weeks of growth. The culture was centrifuged and the supernatant filtered. The filtered material was at pH 8.4. It was clear and brown.

The filtered culture medium was passed over the anion exchange resin. The initial effluent was clear and colorless, and a dark band was formed at the top of the resin column (see Figure 16). With the continued passage of medium over the column the band became more dense in color and greater in height with little tendency to form a second band or smear down the column.

The following fractions were obtained from the anion exchange column:

Fraction 1: This fraction was obtained by pouring the filtered culture medium over the anion exchange column. All of the anions of the growth medium would be removed and exchanged for OH^- groups. Most of the cations in the growth medium would be present in this fraction.

Fraction 2: A water wash to remove residual cations of the medium trapped in the anion exchanger matrix -- this fraction was essentially a dilution of Fraction 1.

Fraction 3: This fraction was obtained by adding acetic acid to the column and it contained some of the anions of the medium which had been exchanged for acetate ions.

Fraction 4: This fraction was obtained by adding formic acid to the medium and it contained additional anions which had been added to or formed by microbial growth in the medium.

Coupons of the aluminum alloy 7075 were placed in each of the fractions obtained from the anion exchange resin. Corrosion tests were run for 18 hours, and controls containing formic or acetic acid showed no evidence of corrosion.

In Fraction 1 definite corrosion was observed, and this corrosion is believed to be caused by the cations of the growth medium which were not adsorbed by the exchanger.

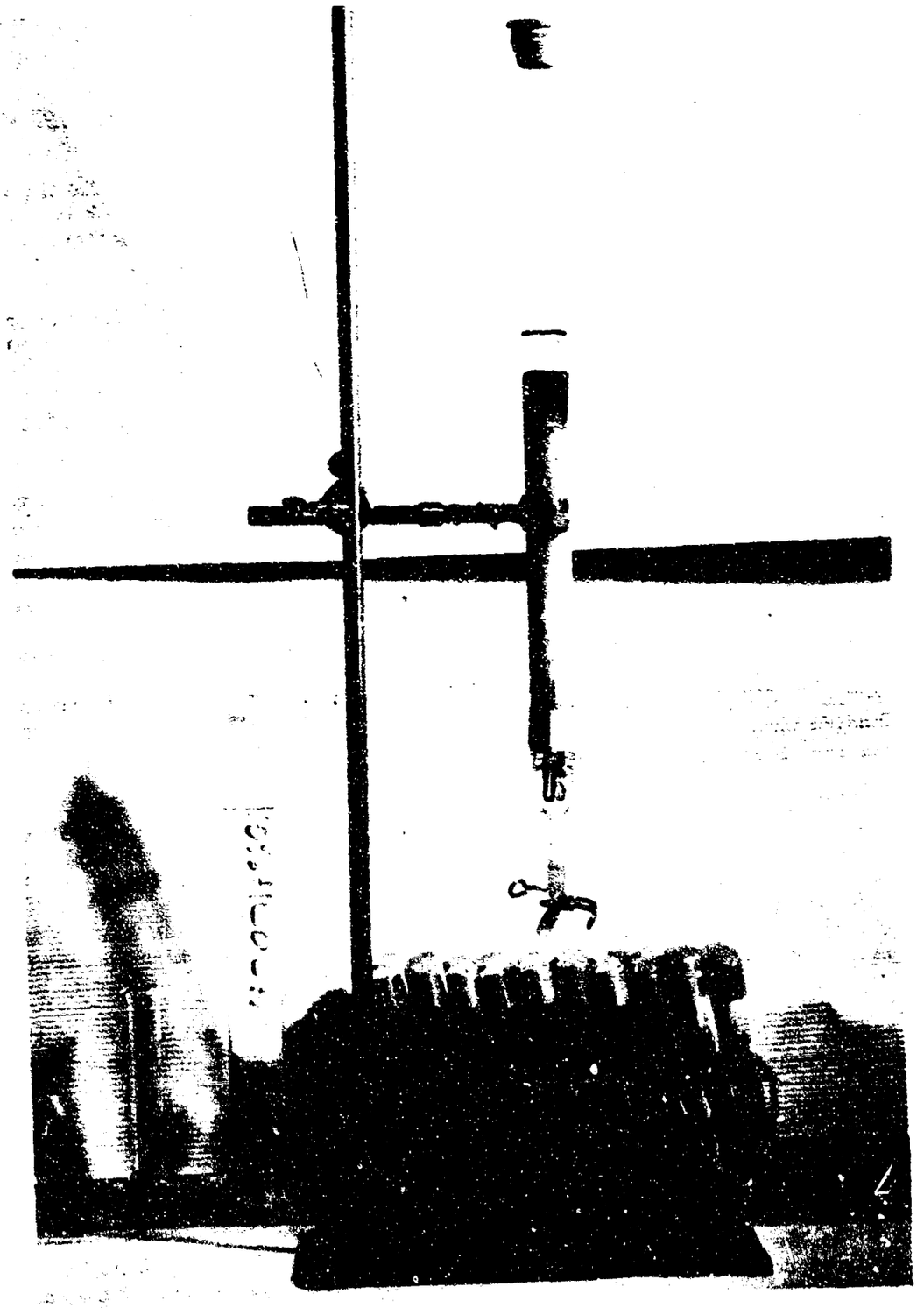


Figure 16. Adsorption of Microbial Products on Dowex-1

In Fraction 2, the water wash, no corrosion occurred.

In Fraction 3, which was faintly yellow, slight corrosion took place.

In Fraction 4, which contained a yellow compound in great concentration, extensive corrosion took place.

Chemical fractions were also obtained from pure cultures of the fuel isolate culture 101. After 49 days of growth, Bushnell-Haas medium was modified by the use of 1.2 g of KNO_3 in place of ammonium nitrate. These cells produced the characteristic yellow compound and the medium became corrosive to aluminum.

This old culture of 101 was subjected to ultracentrifugation at 60,000 X g for 3 hours, at 20°C. The supernatant thus obtained was filtered through a Millipore filter with a 200 mμ pore diameter. The pH of the filtrate was adjusted to 7.0 and then added to a Dowex-1-Cl column. The pigments were again concentrated at the top of the column and they were eluted as shown in Figure 17.

Another portion of the 101 culture was prepared as described above and absorbed on a Dowex-1 column. The portion of the column containing the dark material was removed from the resin bed. The absorbed microbial product was eluted in a batch operation with 10% formic acid. This material was dried. The dried material was subjected to preliminary IR analysis (see Figure 18). Other portions of the eluted fraction are now undergoing C, O, H, and N analyses. The corrosivity of purified fractions of this microbial product will be examined further, and attempts will be made to determine the homogeniety of these products and their molecular weights. This information is desired as a first step in studies of the enzymatic process by which jet fuel contaminants live and by which corrosive compounds are produced.

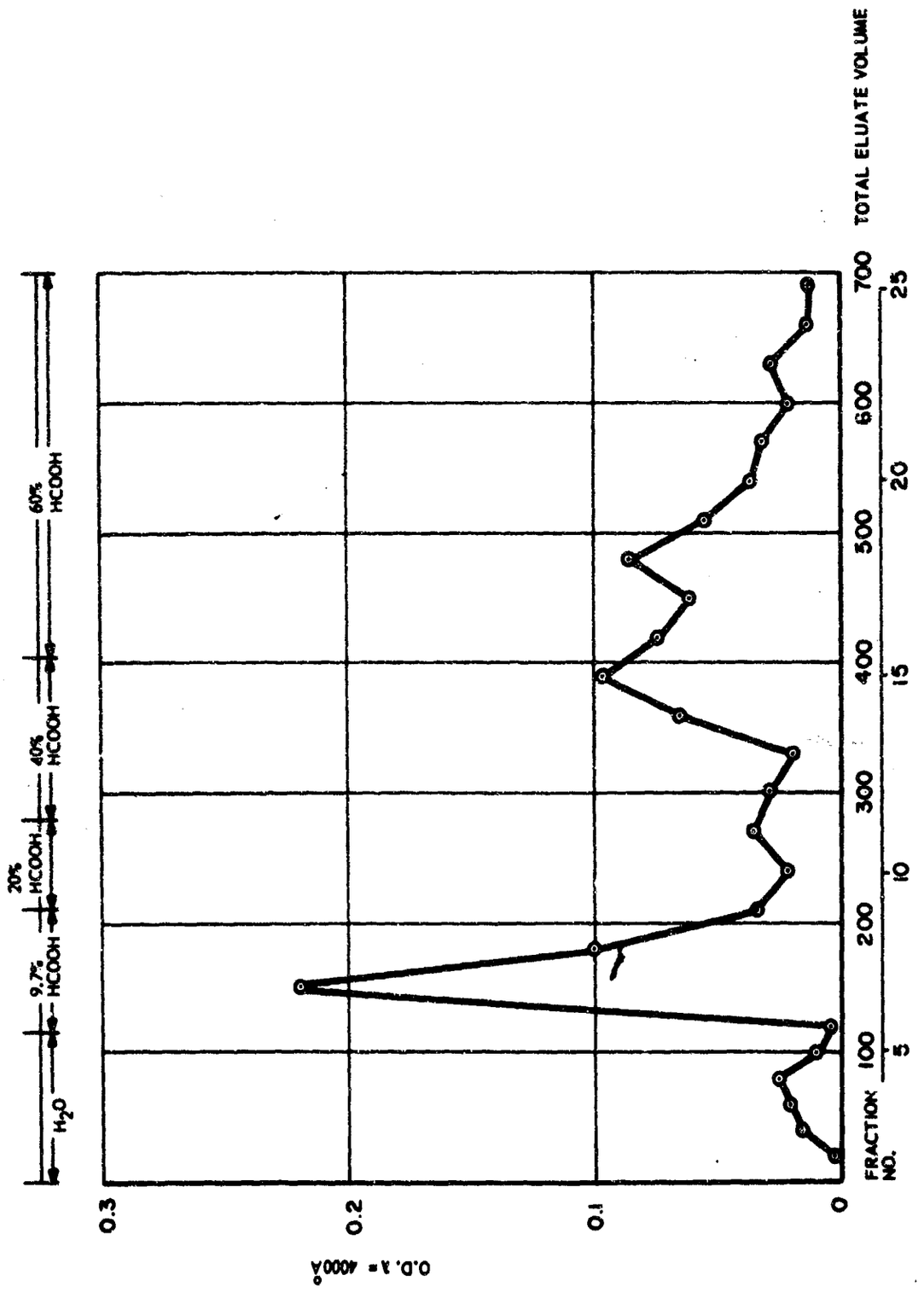


Figure 17. Pattern of Elution of Microbial Products from Dowex-1

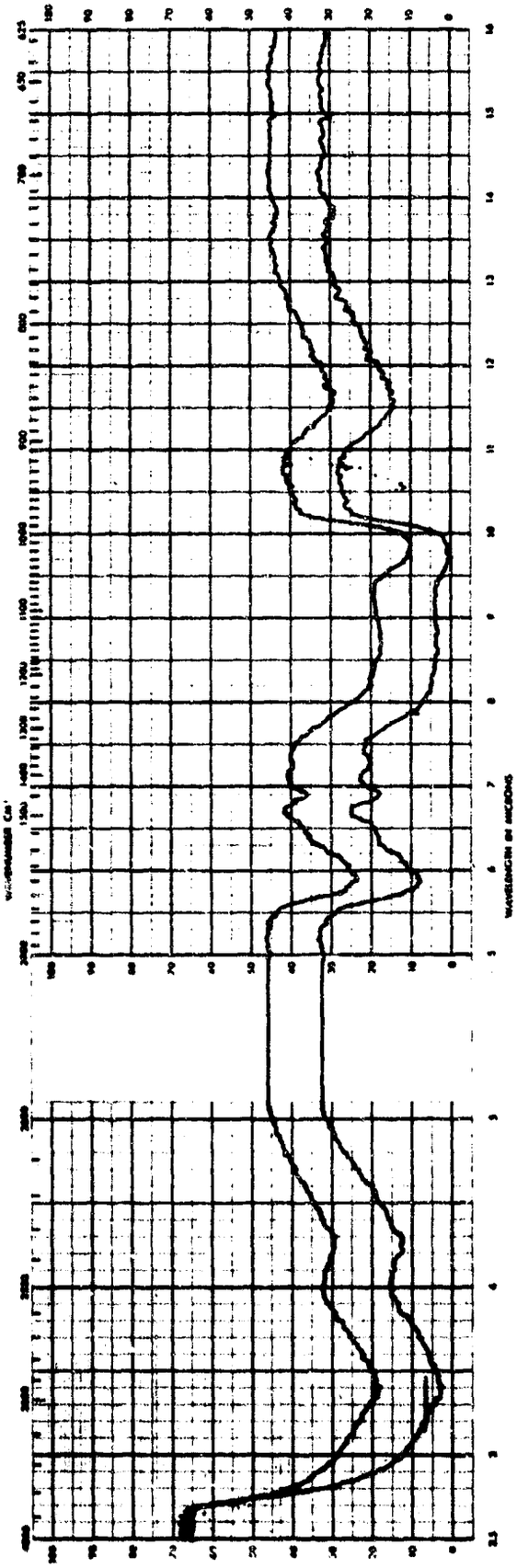


Figure 18. Infrared Spectra of Purified Microbial Products from Old Cultures

C. Jet Fuel Contamination by Metabolic Products of Microorganisms

1. Jet Fuel Penetration and Emulsion Formation by Microorganisms

The ability of microorganisms to penetrate a fuel layer is shown in Figure 19. That this characteristic varied from organism to organism was apparent. In the figure shown, cells of the fuel isolate, culture 96, entered the fuel phase in a short period of time while cells of culture 101, also a fuel isolate, entered the fuel phase only after long growth periods and then only to a small extent. Culture 96 yielded cells that also distributed themselves homogeneously in the aqueous phase of a fuel-water system, but with continued growth the organisms concentrated at the fuel-water interface. These organisms appeared to form an "emulsion," and with old cultures this bacterial mass progressively penetrated the fuel phase.

Studies were performed to characterize the water phase and interface organisms. The latter organisms could not be centrifuged down indicating that their specific gravity had become less than water with continued growth on fuel.

The viability and respiration of top and bottom cells were compared. Difficulties were encountered in making microscopic counts of the top cells because of their tendency to aggregate at the top of an aqueous medium. Nevertheless the cells could be dispersed by vigorous shaking with water, and counting was possible with samples removed immediately after shaking.

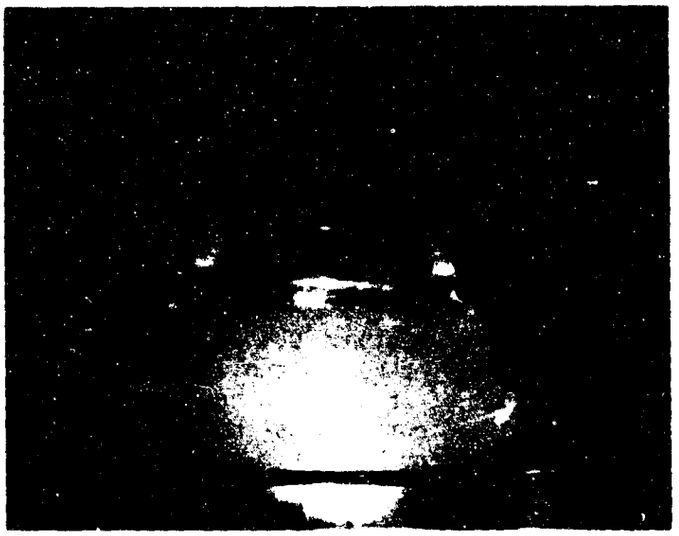
2. Microbial Sludge Formation and Composition

When fuel isolates oxidize JP-4 fuel on a medium containing -- as the only source of nitrogen -- comparatively high concentrations of nitrate, two compounds are produced which have not been observed so far with growth at low nitrate concentrations or with growth on substrates other than jet fuel. One of these compounds was fuel-soluble at hydrogen ionic concentrations that permit microbial growth. The other compound is fuel-insoluble and is also insoluble in water, at least from pH 3 to pH 11. These materials were studied for two reasons: first, they are potential fuel contaminants, and second, they should give some insight into the metabolic function of fuel organisms.

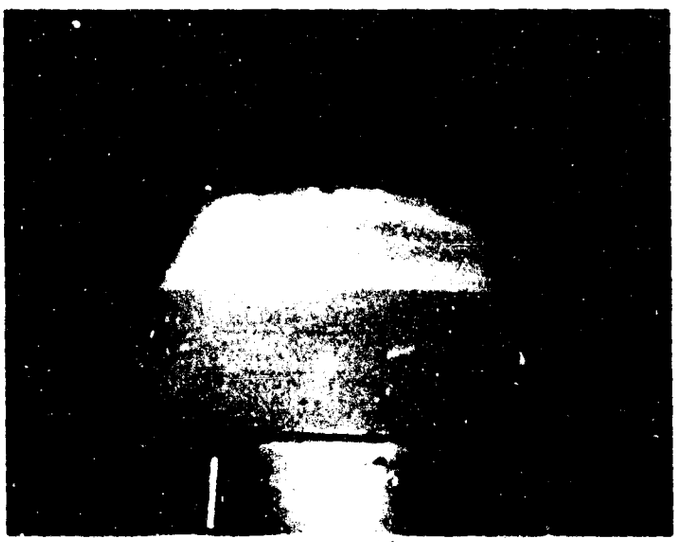
In the analysis, accomplished during this period, an attempt was made to acetylate the microbial sludge by refluxing with acetic anhydride. This treatment resulted in the partial dissolution of the sludge in the acetylating agent. The dissolution of this material may have resulted from the masking of charged groups by acetylation.

The acetylated material, or that material treated with acetic anhydride, was dried and dissolved in methanol with BF_3 as a catalyst. The mixture was refluxed to permit the formation of methyl esters.[6,7] Following this treatment the sludge residue in methanol-BF_3 was dissolved in benzene-ethyl ether.

101



96



LEGEND

THE FERNBACH FLASKS CONTAINED 1 LITER OF NBH MEDIUM AND 200 MLS OF JP-4 FUEL. THE FLASKS WERE INOCULATED WITH 100 MLS OF CULTURE 96 AND 101 RESPECTIVELY. THE TOP FLASK OF 101 AND AND THE BOTTOM FLASK OF 96 ARE REPRESENTED AFTER 48 HOURS GROWTH.

Figure 19. A Comparison of the Ability of Cultures 96 and 101 to Penetrate a Jet Fuel Layer

To this organic solution water was added, and a large quantity of brown material was extracted into the water phase with precipitate formation. The organic phase was separated and washed; it was then analyzed for long-chain methyl esters. The results of that analysis are shown in Table 2. The large proportion of long-chain acids present was of interest.

A portion of the bacterial sludge was further analyzed. It was dialyzed for 24 hours and subject to C, H, and N analyses, Table 3. Another portion (0.104 g) was extracted with 10% HCl-methanol for 48 hours.

The resulting material was separated into a soluble fraction and an insoluble fraction by centrifugation. CHN analyses were performed on these fractions. The weight of the methanol-insoluble fraction actually was greater than indicated because much of it formed a residue on the centrifuge tubes. This residue was not dialyzed out because the residual material in the dialyzed sample was much greater than that present in the extracted sample. The C, H, N, and O values were adjusted to eliminate the effect of this residue. The methanol-insoluble portion contained much more nitrogen than the methanol-soluble portion, with the dialyzed portion having an intermediate value. This was to be expected since the methanol-soluble portion contained, primarily, the lipid portion. The protein concentration was obtained by assuming that normal proteins were responsible for the nitrogen concentration. The residual C, H, and O values were obtained by subtracting the CHO contributions of the protein from the total CHO concentrations. The oxygen value probably has a large error. These results appear to indicate that the protein, lipid, and carbohydrate concentrations, are approximately 50, 20, and 30%, respectively. The presence of nitrogen in this water-insoluble, fuel-insoluble sludge suggests the presence of nitrated hydrocarbons, microbially produced, which contribute to the corrosivity of fuel-water bottoms.

3. Chemical Analysis of Floating Cells

In the course of this study, organisms have been isolated which cause emulsion formation in 24 hours at populations of approximately 10^8 organisms per ml, while other organisms form microbial emulsions at these cell populations after 48 hours or 72 hours of growth. The dependence of emulsion formation on growth conditions suggests that the production of cells that float in water and cause emulsions is an enzymatically controlled reaction, which may cause the slow accumulation of some metabolic product of low specific gravity.

A product with low specific gravity appears to be held within the cell wall or to diffuse from the cell very slowly. In this period, the lipid content of cells which form emulsions and also float were compared with those having a specific gravity greater than water; i.e., those which are dispersed through the growth medium.

The organisms which cause emulsion in a fuel-water system are referred to as top cells; the other water-dispersed organisms are called bottom cells.

TABLE 2

FATTY ACID CONTENT OF MICROBIAL SLUDGE FRACTIONS

Retention Time (minutes)	Peak Height (inches)	Probable Fatty Acid Ester	Percent of Total Material Analyzed
13.34	0.47	14.1	0.09
13.86	0.80	14.1	0.16
14.42	0.25		0.05
17.84	16.88	16.0	3.48
18.98	14.00		2.89
19.44	20.00	17.0	4.12
21.38	2.95	18.1	0.61
21.80	1.02	18.0	0.21
23.24	6.76	19.0	1.39
26.02	0.47	21.1	0.09
26.36	1.73	21.0	0.36
28.50	419.20 (5.24 x 80)	22.0	86.45
32.74	0.38		
Total Height	484.91		99.98

TABLE 3

A CARBON, HYDROGEN, NITROGEN, AND OXYGEN ANALYSIS OF MICROBIAL SLUDGE

	Methanol Soluble	Methanol Insoluble	Dialyzed
Dry weight	0.0322 g	0.0187 g	0.060 g
% C		45.7	25.9
% H		7.8	6.5
% N	4.8	11.3	4.7
% Residue		4.4	39.4
Adj. C		48.0	42.8
H		8.2	10.7
N	4.8	11.9	7.8
O		31.9	38.8
Protein	30.0	74.4	48.8
Residual C		9.4	17.4
H		3.4	7.5
O		15.8	28.0

The analysis of these cells for lipid content was accomplished by taking weighed samples and extracting with acetone in a micro-Soxhlet apparatus for 46 hours.

Portions of the extracts were analyzed by thin-layer chromatography.[8] A 1/2-mm layer of silica gel G was applied to the plates, dried at 100°C for 1 hour, and stored at room temperature. The elution solvent was a mixture of 70% in n-propanol and 30% 1 N NH_4 OH. Approximately 500 µg of each extract were applied to the plates and eluted. After spraying with 3, e'-di-chlorfluorescein, the R_f values were obtained and compared with those of known standards.

Portions of the bacterial extracts were esterified with 10% HCl in methanol at 100°C for 3 hours. After cooling, excess dimethoxypropane was added and the lipids were evaporated to dryness. The resulting fatty acid methyl esters were analyzed in a Beckman GC-2 hydrogen-flame detector gas chromatograph. Table 4 shows the gross lipid content of water bottom cells.

The lipid content of top layer and bottom layer cells appeared to be essentially the same with respect to the general type of lipid present. The top layer cells, however, had about six times more lipid than the bottom layer cells. The phenomenon of flotation was associated with the production of large quantities of lipids which were normal to these fuel isolates. The emergence of top layer emulsion-forming cells seemed to result from the utilization of metabolic pathways common to both types of organisms.

4. Fuel Contamination by Metabolic Products

In general, in this laboratory the fuel isolates that have been grown in Bushnell-Haas medium with a jet fuel overlay have not produced colored compounds that are soluble in jet fuel. Fuel extractable compound(s) were produced, however, in cultures grown at a high-nitrate concentration with nitrate as the only source of nitrogen.

The compound produced under these conditions was yellow; the absorption spectrum of this compound in fuel and in water at two extremes of pH is shown in Figure 20. The compound entered the fuel layer at pH 7 and was easily extracted into water and concentrated by adjusting the pH to 11.5. In aqueous solution, the compound showed a definite inflection point as a function of pH; hence, it has a weak acidic functional group which exercises an inductive effect on its chromophore. Advantage was taken of the pH dependence of the extinction coefficient, and a spectrophotometric titration of the compound was made. For this titration, a mixed buffer containing 0.1 M Tris and NaOH, adjusted to the indicated pH level with HCl, was used. Figure 20a shows this determination. The clearly defined inflection point at pH 10.8 sets the apparent pK of the functional groups which control color change.

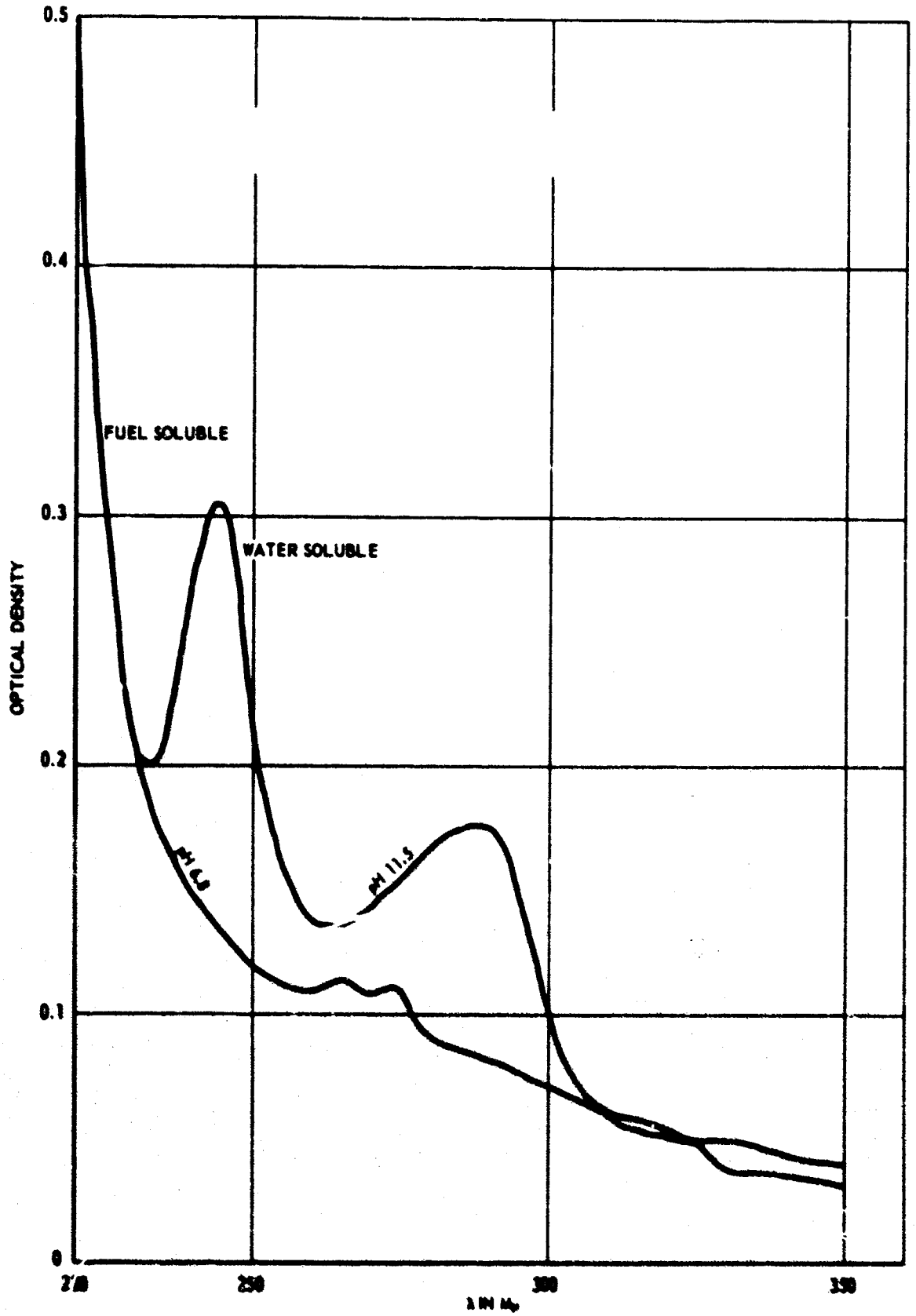


Figure 20. The absorption Spectra of a Fuel Extractable Compound at pH 6.8 and pH 11.5

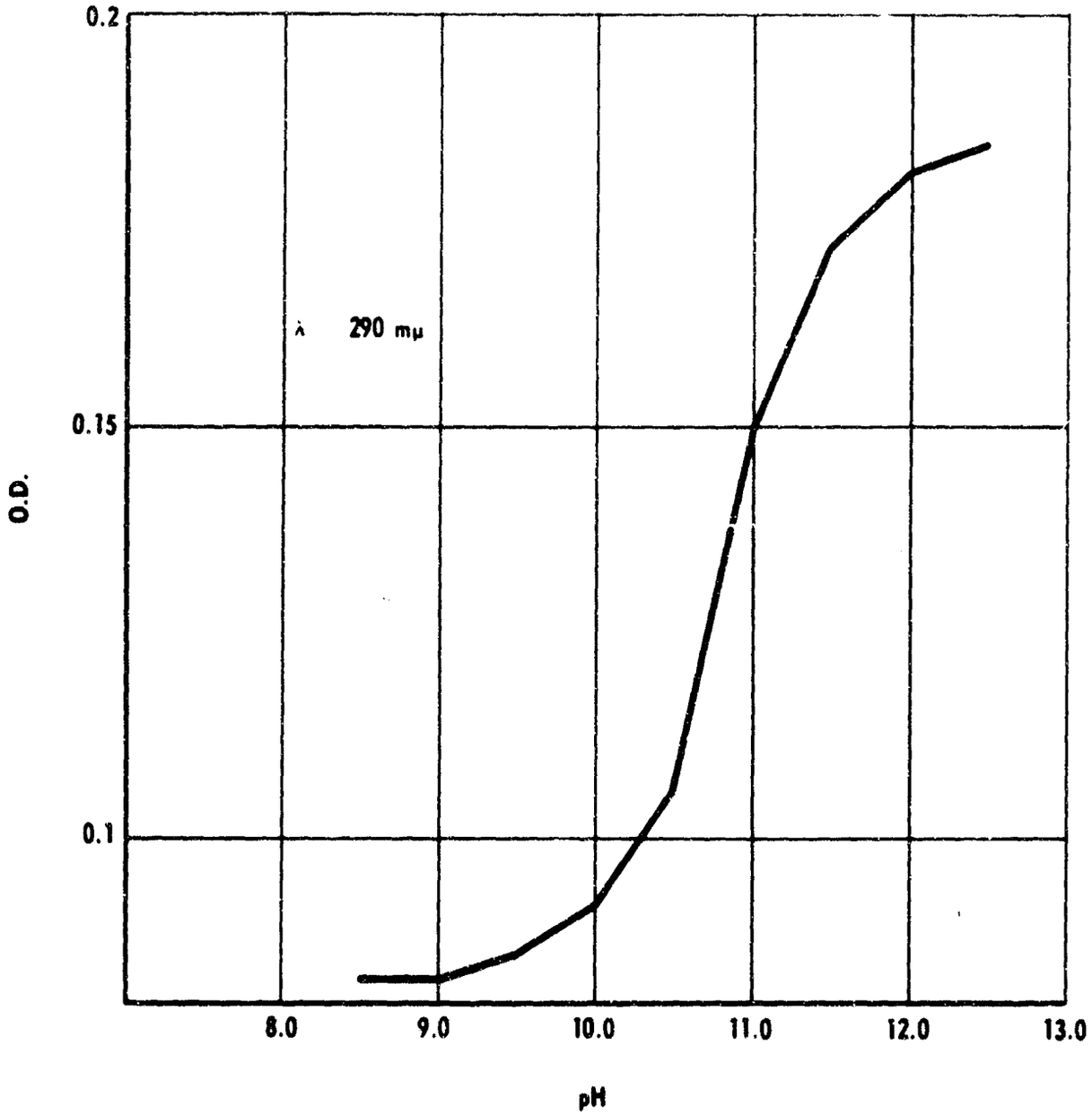


Figure 20a. The Spectrophotometric Titration of a Fuel Extractable Compound

TABLE 4

A COMPARISON OF LIPID CONTENT OF TOP AND BOTTOM CELLS

	Top Layer	Bottom Layer
Weight of Bacteria	60.5 mg	269.1 mg
Weight of Extract	20.7 mg	14.6 mg
% Lipid Extract	34.0%	5.4%
Type of Lipids	Equal amounts of phospholipids, monoglycerides, triglycerides	Equal amounts of phospholipids, monoglycerides triglycerides

It seems probable that this group also controls the water solubility of the compound. Hence, these determinations show that this microbially produced fuel contaminant will begin to enter the fuel layer when the aqueous hydrogen ion concentration diminishes to values which permit microbial growth.

D. Biochemical Activities of Fuel Isolates

1. The Growth and Viability of Fuel Isolates in Media Containing Purified Hydrocarbons

Many microorganisms which are capable of oxidizing hydrocarbons are capable of oxidizing the five-carbon hydrocarbon pentane. The organisms isolated from JP-4 fuel in this study, however, were unable to oxidize pentane. In an effort to characterize these organisms and their metabolic products it was necessary to determine their capacity to grow on hydrocarbons of different chain length and structure. Table 5 summarizes the responses of microorganisms in the presence of the particular alkanes and olefins used in growth media.

Pentane, hexane, and heptane do not support growth, and they do not kill the fuel isolates tested. The organisms are not killed by 1-pentane and are killed only slowly by 2-pentene. However, 1- or 2-hexene or heptene, 1- or 2-octene or nonene kill these fuel isolates readily. But the effectiveness of these short chain olefins end with nonene; and either 1-decene or 1-dodecene support growth to about the same extent as octane, nonane, decane, and dodecane. Population densities change on these saturated hydrocarbons from 10^6 cells per ml to about 10^8 cells per ml in 48 hours.

The toxicity of short-chain olefins is not confined to organisms that grow on fuel. Table 6 shows that E. coli is distinguished from the pseudomonads isolated from fuel by its sensitivity to jet fuel, but both organisms are sensitive to the lethal properties of 1-heptene.

The response of these fuel isolates to short chain olefins has no explanation in known physiological or biochemical mechanisms. It is surprising that a nine carbon hydrocarbon with one double bond is both a respiratory inhibitor and a biocide, while the same compound without unsaturation acts as a carbon source for growth.

Information is sought as to the physiological locus of action of these olefins. Does death by olefins result from respiratory inhibition, or do they interfere in some very specific way with the cell division and reproduction. Future work on the enzymes and growth characteristics of fuel isolates will attempt to answer these questions.

2. The Respiration of Fuel Isolates with 5 to 10 Carbon Alkanes, and Alkenes, and Unsaturated Cyclic Hydrocarbons

The failure of fuel isolates to grow on short-chain alkanes and olefins prompted a study of the effects of these short-chain hydrocarbons on the metabolic pathways essential to the life of the organism.

TABLE 5

THE GROWTH AND VIABILITY OF A FUEL ISOLATE IN MEDIUM CONTAINING PURIFIED HYDROCARBONS

Hydrocarbon	Viable Response
Pentane	No growth
1-Pentene	No Growth
2-Pentene	Kills
Hexane	No Growth
1-Hexene	Kills
2-Hexene	Kills
Heptane	No Growth
1-Heptene	Kills
2-Heptene	Kills
Octane	Growth
1-Octene	Kills
2-Octene	Kills
Nonane	Growth
1-Nonene	Kills
Decane	Growth
1-Decene	Growth
Dodecane	Growth
1-Dodecene	Growth

Legend: The organism tested was the fuel isolate Culture 101. These cells were grown on BH medium with a fuel overlay. They were harvested by washing 3 times in water and the washed cells were used as inocula in media overlayed with the purified hydrocarbons shown above.

TABLE 6

THE EFFECT OF JP-4 FUEL ON THE VIABILITY OF E. COLI AND A FUEL ISOLATE

Inoculum	Media	Initial Count	Percent Survival		
			0 Time	2 days	4 days
	Distilled H_2O	2.8×10^8	100%	0.075%	0.0679%
E. coli	NBH-fuel	3.6×10^8	100%	0.75%	0.01%
96	Distilled H_2O-Fuel	2.14×10^7	100%	91.6%	78.5%
	NBH-fuel	1.98×10^7	100%	51%	17.18%
96	Distilled H_2O-No fuel	1.3×10^6	100%	146%	615%
E. coli	Distilled H_2O-No fuel	3.8×10^8	100%	105.5%	150%
E. coli	Saline-fuel	3.67×10^8	100%	0.0329%	0.039%
	NBH-fuel	2.27×10^8	100%	0.0538%	0.031%
96	Saline-fuel	3.1×10^7	100%	10.32%	8.9%
	NBH-fuel	1.65×10^7	100%	16.96%	13.34%
E. coli	Saline - no fuel	2.5×10^8	100%	52%	76.9%
96	Saline - no fuel	1.1×10^6	100%	276%	255%
96 (Saline washed cells)	Saline-fuel	6.0×10^2	100%	966.6%	7,166.6%
	NBH-fuel	4.0×10^2	100%	20,000%	73,333%
96 (Distilled H_2O washed cells)	Distilled H_2O - fuel	4.5×10^2	100%	3111%	7333%
	NBH-fuel	2.2×10^2	100%	42,636%	190,909%

As shown previously, octane and nonane are readily oxidized by fuel isolates and they also support microbial growth, but octene and nonene, like hexene and heptene, are inhibitory to the oxidation of jet fuel and glucose. The response to these olefins is in sharp contrast to that of compounds containing one additional carbon atom, namely 1-decene. This unsaturated compound is capable of supporting growth and it is inhibitory neither to jet fuel oxidation nor to glucose oxidation. The same pattern of response is observed with 1-dodecene which supports growth and does not cause respiratory inhibition.

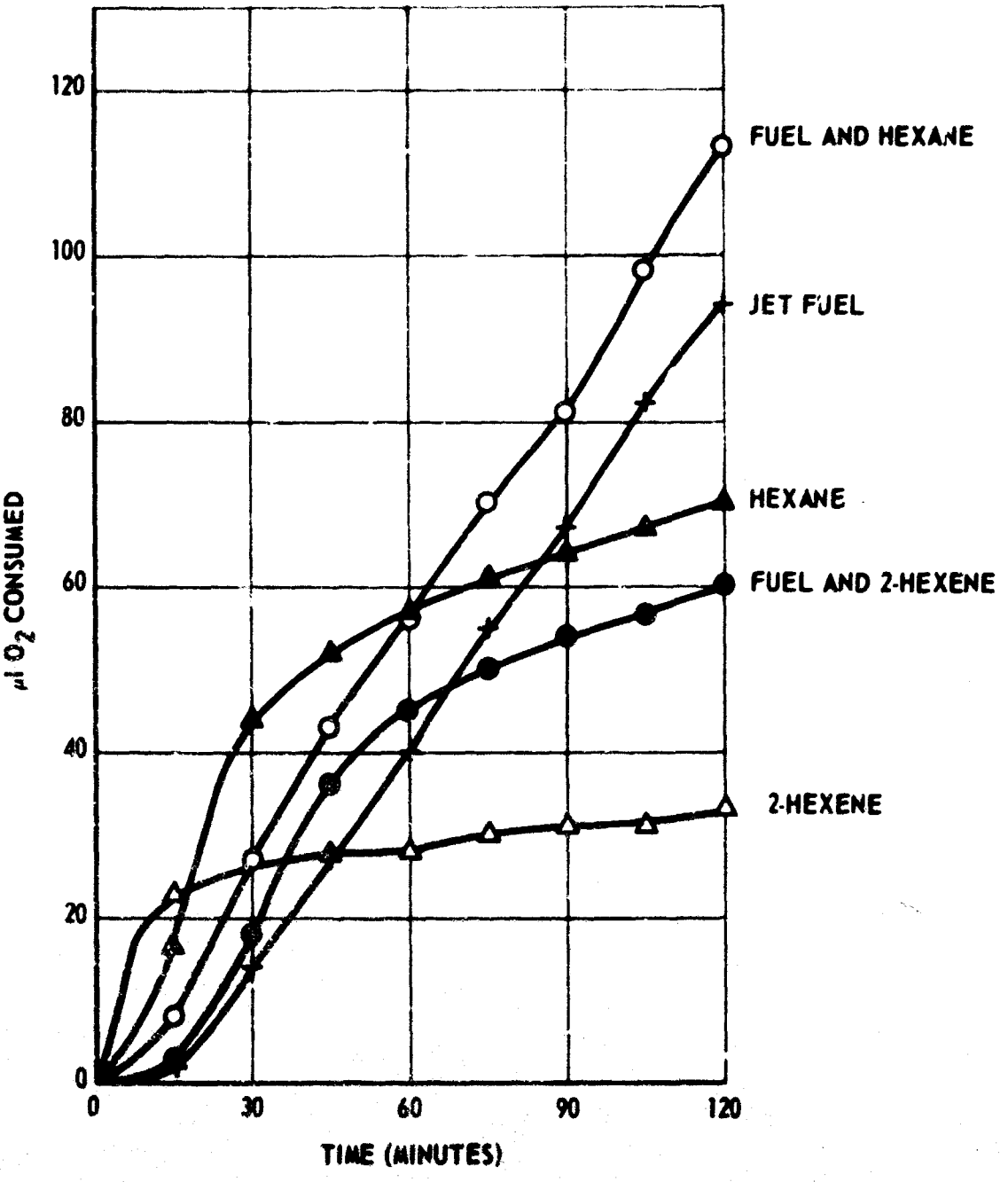
Table 7 summarizes our experience with short-chain hydrocarbons and their ability to undergo oxidation or to affect respiratory inhibition. A knowledge of the site of action of the unsaturated hydrocarbons which inhibit should be of considerable importance in understanding the physiological mechanisms by which microorganisms oxidize fuel and contribute to the formation of microbial sludges and emulsions.

The typical effect of short chain unsaturated hydrocarbons on jet fuel oxidation is shown in Figure 21. The inhibitition of glucose by 2-hexene assumes essentially the same time course as that shown for jet fuel oxidation. The respiration of these fuel isolates on both jet fuel and glucose is also profoundly affected by unsaturated wing structures such as p-xylene. (Figure 22.). These data show the difficulty of formulating general statements about the effect of hydrocarbons on the respiration of cells isolated from fuel systems. The inhibitory effect of hydrocarbons appears to be associated both with unsaturation and with chain length.

3. The Effect of Known Respiratory Inhibitors on Microbial Oxidation of Jet Fuel and Purified Hydrocarbons

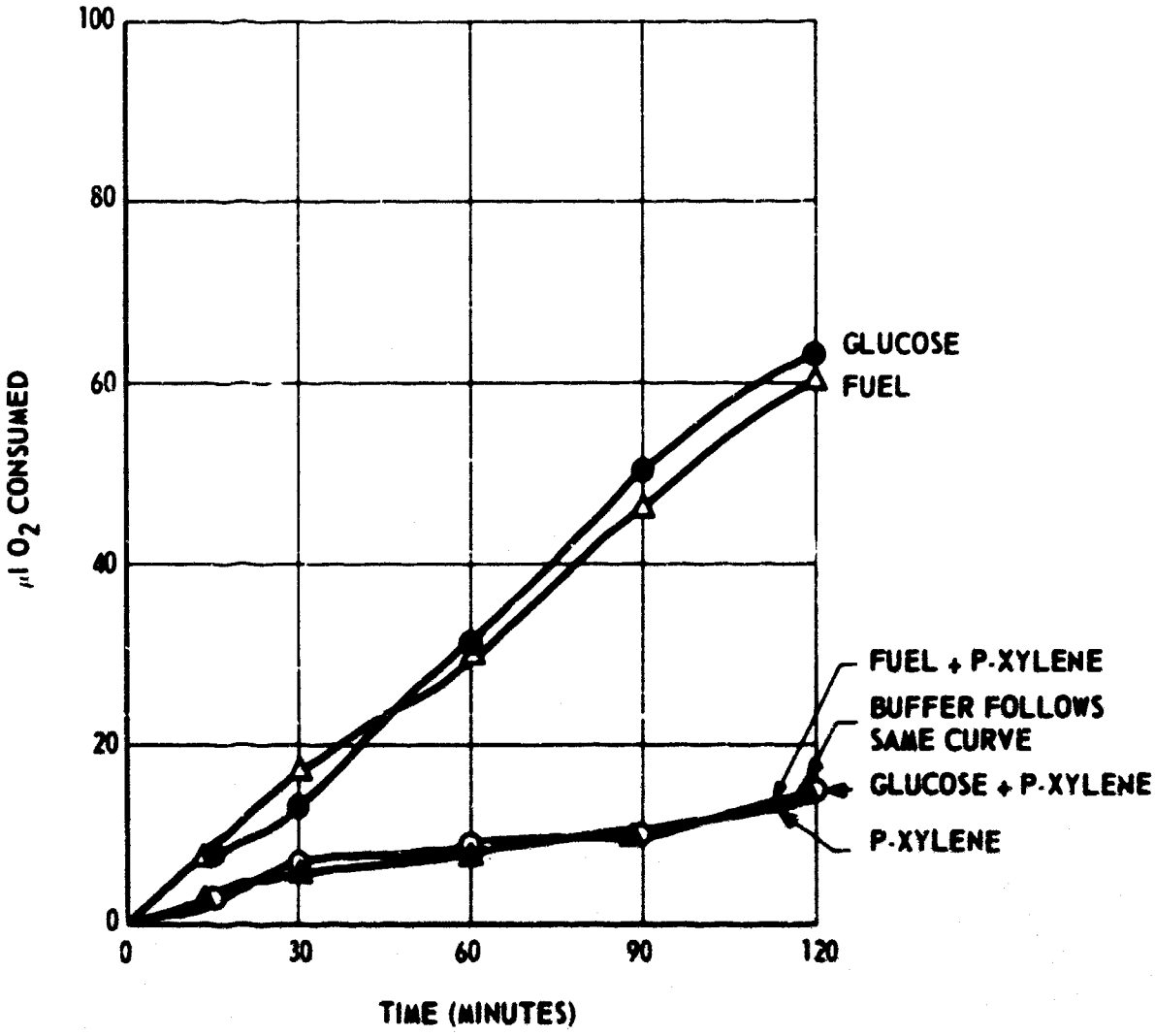
The character of metabolic mechanisms responsible for particular chemical transformations carried out by microorganisms can frequently be elucidated by the use of compounds which inhibit normal physiological activities. The application of this approach (form of reasoning) to an understanding of the physiological mechanisms operative in jet fuel oxidizing organisms has been attempted. The metal requirement shown by these studies reported for in vitro conditions in the literature[10] suggest that the enzymes responsible for fuel oxidation and bacterial respiration contain metals. Sodium azide inhibits the activity of metal containing enzymes and, like 2,4-dinitrophenol, it prevents adaptation.

Tests were made of the effect of respiratory inhibitors on the activity of fuel isolates. The anabolic inhibitor, 2,4-dinitrophenol which stops adaption, is effective at concentrations as low as 10^{-5} M against some organisms. The data in Figure 23 show that concentrations of this compound as high as 10^{-3}M do not appreciably affect the ability of jet fuel organisms to oxidize hydrocarbons. Sodium azide might be expected to react with the metal in enzyme systems oxidizing hydrocarbons. This reaction seemed



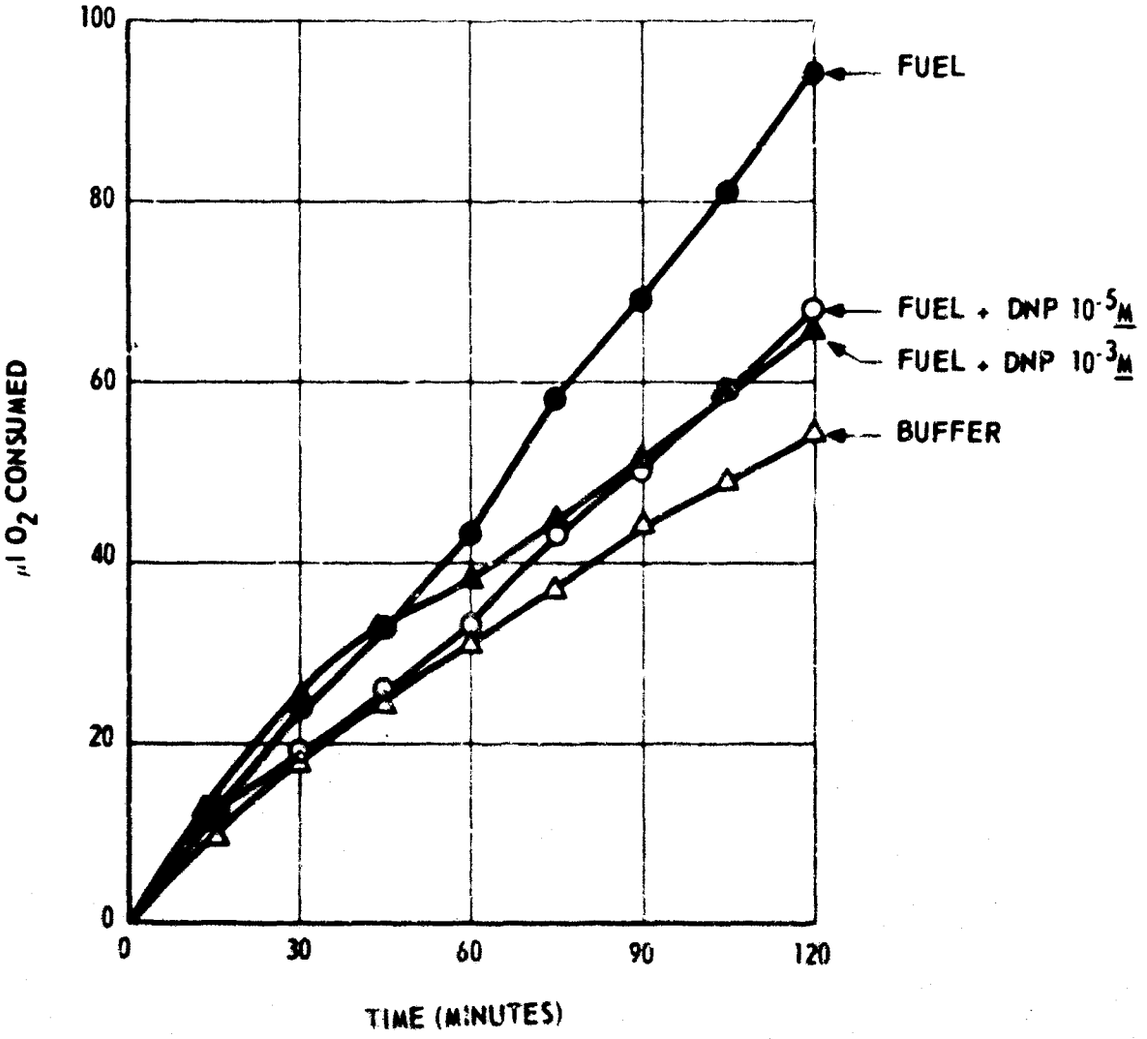
LEGEND: FLASK CONTENTS: 1.0 ML CELLS (1.4 X 10^{10}/ML); 1.0 ML 10^{-2}M PHOSPHATE BUFFER pH 7.1, 0.5 ML FUEL OR GLUCOSE; 0.5 ML HEXANE OR 2-HEXENE OR BUFFER; CENTER WELL 0.2 ML 25% KOH. REACTION RUN AT 37°C.

Figure 21. The Effect of Hexene on the Oxidation of Jet Fuel Culture 101



LEGEND: FLASK CONTENTS: 1.0 ML CELLS (9.2×10^9 /ML); 0.5 ML GLUCOSE OR FUEL; 1.0 ML 10^{-2} M PHOSPHATE BUFFER pH 7.1; 0.5 ML P-XYLENE OR BUFFER; CENTER WELL 0.2 ML 25% KOH. REACTION RUN AT 37°C.

Figure 22. The Effect of P-Xylene on the Oxidation JP-4 Fuel and Glucose by Culture 101



LEGEND: FLASK CONTENTS: 1.0 ML CELLS (2.1×10^{10} ML 0.5 ML FUEL; 1.0 ML 10^{-2}M PHOSPHATE BUFFER pH 7.1, 0.5 ML DNP (6×10^{-3} M); CENTER WELL 0.2 ML 25% KOH. REACTION RUN AT 37°C.

Figure 23. The Effect of 2,4-Dinitrophenol on Jet Fuel Oxidation by Jet Fuel Isolates

TABLE 7

THE RESPIRATION OF STRAIN 101 IN THE PRESENCE OF SHORT-CHAIN SATURATED AND UNSATURATED HYDROCARBONS

Hydrocarbon	Oxygen Uptake	Effect on Fuel Oxidation	Effect on Glucose Oxidation
Pentane	No	Slight Inhibition	Slight Inhibition
1-Pentene	No	Inhibition	Inhibition
2-Pentene	No	Inhibition	Inhibition
Hexane	Variable: Always Small	Slight Inhibition	Slight Inhibition
1-Hexene	No	Inhibition	Inhibition
2-Hexene	Variable: Always Small	Inhibition	Inhibition
Heptane	Not Sustained	No Inhibition	No Inhibition
1-Heptene	Not Sustained	Inhibition	Inhibition
2-Heptene	No	Variable, Inhibited	Inhibition
Octane	Yes	No Inhibition	No Inhibition
1-Octene	No	Inhibition	Inhibition
Nonane	Yes	No Inhibition	No Inhibition
1-Nonene	No	Inhibition	Inhibition
Decane	Yes	No Inhibition	No Inhibition
1-Decene	Yes	No Inhibition	No Inhibition
Dodecane	Yes	No Inhibition	No Inhibition
1-Dodecene	Yes	No Inhibition	No Inhibition

especially probable since the metal involved appeared to be iron, but here again the metabolic activity of the fuel isolates proved to be surprisingly atypical and resistant. Even 10^{-3} M azide inhibited respiration only 30% (Figure 24.) This cellular response indicates that the terminal oxidase may be a flavin-containing enzyme rather than a cytochrome.

This study of metabolic mechanisms emphasizes the complexity of factors controlling the appearance and activity of fuel contaminants. The results obtained show the presence of areas of unexpected resistance and sensitivity in the physiological makeup of organisms which inhabit fuel-water systems.

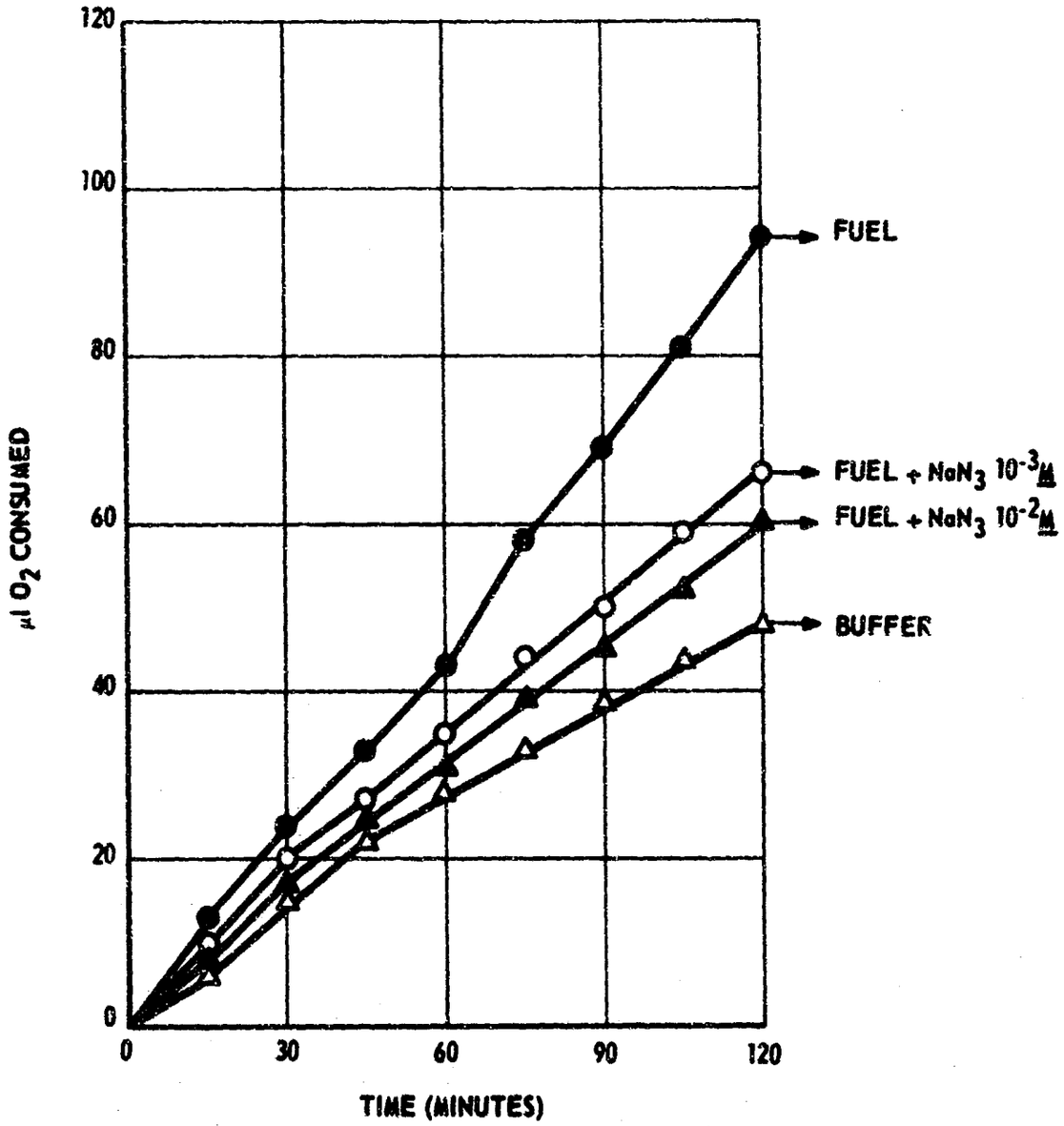
4. The Killing of Fuel Isolates by Hydrocarbons

It was hypothesized that fuel-oxidizing cells entered fuel systems either as cells adapted to growth on rich media or as cells adapted to growth on fuel-containing media, while the fuel-water bottom may possess a mineral content ranging from predominatly sodium chloride to that of a complete growth medium in terms of trace metals and nitrogen source. In this phase of the study fuel-oxidizing organisms (Culture 96) were grown both on a modified Bushnell-Haas medium with a fuel overlay and on TGY. The cells were harvested by centrifugation and were washed and suspended in either NaCl solution or H_2O. The washed cells were then inoculated into NaCl solution, H_2O, or BH-medium with a fuel overlay. Cells in each medium grew during exposure to fuel for 4 days.

The results obtained (Table 6) show the extreme hardiness of the organisms which oxidize fuel, and although a limited sample was analyzed, the viability of this particular fuel-oxidizing organism did not appear to be strongly dependent on the kind or concentration of salts in the water phase of its environment. It was observed that a very large fraction of these cells grown on TGY survive when placed in the presence of fuel.

Cells of Escherichia coli were grown on TGY (trypticone-glucose-yeast extract) medium and treated in every way similar to the fuel isolates. In 4 days the viability of the E. coli suspension diminished to 0.03% to 0.06% of its initial value, but the viability of Culture 96 remained essentially unchanged during this period (Table 6.) The viability of E. coli and Culture 96 in saline and distilled water without fuel was essentially unchanged during this 4-day period. Esentially the same results were obtained with nitrogen deficient medium where the cell count remained constant in absence of growth for 97 days. These results show that fuel components are toxic to the nonfuel-oxidizing organism, E. coli, but not to fuel organisms.

The reason for these differences in physiological terms is not immediately apparent. The Gram-stain response separates microorganisms into two great classes in terms of the complexity of cell membrane structure. But in this study both E. coli and the Psuedomonads are gram negative, but their grossly different rate of survival in water-hydrocarbon systems demonstrates



LEGEND: FLASK CONTENTS: 1.0 ML CELLS (2.1 X 10^{10} ML); 0.5 ML FUEL; 1.0 ML 10^{-2} PHOSPHATE BUFFER pH 7.1; 0.5 ML NaN_3 (6 X 10^{-3} M) OR PHOSPHATE BUFFER CENTER WELL 0.2 ML 25% KOH. REACTION RUN AT 37°C.

Figure 24. The Effect of NaN_3 on the Oxidation of Jet Fuel by Jet Fuel Isolates

that a further distinction may be made among Gram-negative organisms. It is believed that this distinction derives from difference in permeability to hydrocarbon molecules.

E. The Effect of Fuel Additives on Microorganisms and on Aluminum Corrosion

Tests were made to determine the ability of organisms isolated from fuel to survive or grow in the presence of fuel additives such as anti-oxidants and anti-icing compounds. Other long-term tests were set up to study the effect of antioxidants on the corrosion caused by large concentrations of microbes and, on the aluminum corrosion caused by frequently encountered fuel contaminants such as rust.

For testing effects of fuel additives on microbial growth, media were prepared consisting of 100 ml of Bushnell-Haas medium with a 10 ml JP-4 overlay. To this fuel overlay, from 0.05 ml to 10 ml of fuel additive was added. Viable cell counts were made periodically up to 24 hours.

If antioxidants are generally present in fuels, then it is probable that they affect the metabolism of fuel organisms and enter into corrosion and fuel system deterioration caused by other fuel contaminants. The response of fuel isolates to fourteen of the antioxidants was tested and found to be essentially identical, with two notable exceptions. (The exceptions are discussed below.) The compounds received are listed in Table 8 together with the concentration used in the fuels, and the gross effect on microbial survival.

Both antioxidants and corrosion inhibitors affected microbial growth at low concentrations. In Bushnell-Haas medium as little as 1 mg per ml of additive influenced microbial growth and survival. The antioxidants were, in general, bactericidal; however, they killed organisms at a much slower rate than the olefins tested earlier. Lubrizol 541, a corrosion inhibitor, and Lubrizol 802, an antioxidant, were the most bactericidal compounds among the group tested. (See Table 9.) Tolad 244 did not kill the fuel isolates studied; and the corrosion inhibitor Unicor M supported microbial growth.

With Unicor M as the only source of carbon in Bushnell-Haas medium the number of organisms present increased from 10^6 to 10^8 cells per ml in 24 hours. (See Table 10.) It is possible that this compound is a potential source of nitrogen for organisms that oxidize fuel.

TABLE 8

MICROBIAL SURVIVAL IN THE PRESENCE OF FUEL ADDITIVES

Compound	R	%S
Unicor M	0.10	>8815.0
Tolad 244	0.10	<250.7
MBX-200	0.10	<88.7
Ethyl AN 33	0.05	<1.02
Dupont HP2	0.05	<0.38
Lubrizol 802	0.01	<0.001
Santolene C	0.10	<0.01
Metal Deactivator	0.01	<0.01
Lubrizol 541	0.05	<0.01

Legend: $R = \frac{\text{ml additive}}{\text{ml NBH}}$

%S = (Viable cell count after 24 hours/initial viable count) x 100. All viable counts were made after 24 hours exposure at 30°C. The indicated concentrations of additive were added to Bushnell-Haas medium. Each medium was inoculated with 5 mls of culture 101 grown on fuel. The cells had been washed 3 times in distilled water. Viable counts were made in TGY agar at periodic intervals for 24 hours. Incubation: 30°C.

TABLE 9

EFFECT OF ANTIOXIDANT LUBRIZOL 802 ON GROWTH OF CULTURE 101

ml of additive per 100 ml NBH	0 hr	% Survival 0 hr	2 hrs	% Survival 2 hrs	24 hrs	% Survival 24 hrs
0 ml	1.40×10^{7}	100	6.2×10^{6}	44.28	1.62×10^{7}	115.71
0.05 ml	1.29×10^{7}	100	1.05×10^{5}	1.27	no growth	0.00
0.1 ml	3.1×10^{6}*	23.84	2.8×10^{5}	2.15	no growth	0.00
1.0 ml	5.7×10^{5}*	4.39	3×10^{2}	0.0023	no growth	0.00
5.0 ml	3×10^{1}*	0.00023	no growth	0.00	no growth	0.00
10.0 ml	no growth	0.00	no growth	0.00	no growth	0.00

*innoculated with 1.3×10^{7} cells.

Legend: See Table 8.

TABLE 10

EFFECT OF CORROSION INHIBITOR UNICOR "M" ON GROWTH OF CULTURE 101

ml additive per 100 ml MBM	0 hr	% Survival 0 hr	2 hr	% Survival 2 hr	4 hr	% Survival 4 hr	6 hr	% Survival 6 hr	24 hr	% Survival 24 hr
0 ml	$1.37x10$	100	$9.7x10^{5}$	70.80	$1.10x10^{7}$	80.20	$1.26x10^{7}$	91.97	$1.99x10^{}$	145.25
0.05	$1.29x10^{7}$	100	$133.1x10^{7}$	103.10	$1.34x10^{7}$	103.87	$1.17x10^{7}$	90.69	$2.51x10^{7}$	194.47
0.1	$9.6x10^{6}$	100	$9.9x10^{6}$	103.12	$1.09x10^{7}$	113.54	$1.34x10^{7}$	139.58	$2.31x10^{7}$	240.62
1.0	$9.3x10^{6}$	100	$9.1x10^{6}$	97.84	$1.13x10^{7}$	121.50	$1.27x10^{7}$	136.55	$7.2x10^{7}$	774.19
5.0	$8.0x10^{6}$	100	$9.9x10^{6}$	123.75	$8.7x10^{6}$	108.75	$1.36x10^{7}$	170.00	$5.1x10^{8}$	6375
10.0	$7.6x10^{6}$	100	$1.29x10^{7}$	169.73	$1.30x10^{7}$	171.05	$9.0x10^{6}$	118.42	$6.7x10^{8}$	8815.78

Legend: See Table 8.

F. The Physical and Chemical Characteristics of Cultures of Jet Fuel-Oxidizing Microorganisms

1. The Size and Appearance of Microbial Products which Clog Filters

Reports from other laboratories have indicated that filters and pumps of Air Force jet fuel systems have been clogged by microbial growth. In the period reported on, a study was made of the physical and chemical characteristics of microbes and microbial products which appeared to be capable of causing such difficulties in fuel systems.

Observations were made of cultures of fuel-oxidizing organisms "grown" for 3 to 6 months on a modified Bushnell-Haas medium with a JP-4 jet fuel overlay. These cultures were white and the jet fuel overlay was essentially colorless during the first month of growth; the cultures were also noncorrosive. Following this period and up to the third month, the color of the culture darkened, the jet fuel overlay became progressively more yellow, and the cultures became corrosive.

The cells in a 3-month-old culture which was initially high in nitrate were, for the most part, sedimented in centrifugal fields of moderate strength (30,000 X gravity). The clear supernatants thus obtained were corrosive to the aluminum alloy 7075. If growth was permitted to continue, the corrosivity of the medium was enhanced and the general appearance of the culture was significantly changed. Old cultures of fuel-grown organisms, 3 to 6 months in age, became murky and brown. The organisms which initially possessed a recognizable morphology became pleomorphic. Microscopically, the contents of such cultures were not distinquishable as microorganisms but appeared as particles of different dimensions.

In order to make chemical analysis of old cultures of fuel-grown microorganisms, attempts were first made to separate physically the culture content. These cultures were subjected to a centrifugal force of about 30,000 X gravity for 1 hour. Only a very small sediment formed and an exceedingly small pellicle or fluff was present at the air-water interface.

The behavior of these relatively large particles in the centrifuge suggested that they may be lipid in character and that they may be eliminated either by filtration or by extraction with organic solvents.

Attempts to filter the particles led to the belief that such microbial products may indeed be the entities responsible for filter clogging in jet fuel systems. Attempts were made to filter the old culture through a variety of filters following 30,000 X gravity centrifugation. Millipore filters of 1.2 micron porosity did not retain the particles, and filters of 0.8 micron porosity retained both the particles and the suspending fluid. These products of the biodegradation of fuel appeared to be unusually capable of clogging filters and while they have dimensions possibly less than 0.8 micron they prevent fluid passage as well as particle passage through 0.8 micron filters.

The particles described are a product of microbial metabolism of jet fuel. They do not appear in old cultures of fuel-oxidizing organisms which have been grown on rich media such as TGY in the absence of fuel.

2. The Distribution of Filter Clogging Microbial Products in Various Solvent Systems

The suspension formed in old cultures of fuel isolates were believed to be lipid in character because of their resistance to sedimentation, and because of their tendency to coalesce and prevent filtration. In order to remove the particles (or globules) for analysis, attempts were made to extract old cultures with hexane, benzene, ethyl ether, and chloroform. The dielectric constants of these solvents are, respectively, 1.82, 2.28, 4.33, and 4.8; thus, materials might be separated having a modest range of polarities.

Systems were prepared in separatory funnels consisting of equal portions of a 6-month-old culture medium and an organic solvent. The jet fuel was removed from the old culture before analysis. It was surprising to find that hexane, benzene, and chloroform did not extract colored material from the old cultures, nor did they appear to affect the suspended material in these cultures. In contrast, ethyl ether caused the suspended material to go into solution almost immediately. Although the pH of the medium was near 7, the microbial products in the old culture entered the ether phase as a yellow solute and the aqueous phase became clear and yellow. A small white fluff appeared at the water-ether interface.

It would appear that the ether was able to break a complex between a water-soluble and a lipid-soluble material because the total color component in the medium diminished more than would be anticipated by simple dilution or extraction with the quantity of ether used.

It is speculated that the ether may have separated the lipid from a lipoprotein or a lipopolysaccharide. The extraction of a yellow compound by an organic solvent at pH 7 has not been frequently observed in this study. Tests will be made of the ability of the ether-extractable fraction, which is a potential fuel layer contaminant, to cause aluminum corrosion. If the compounds of this fraction are corrosive, further analysis will be made in an attempt to identify the active groups of the compound which cause corrosion.

In regard to the work on old cultures and their character, it should be pointed out that the laboratory-prepared cultures maintained for 3 to 6 months grossly resemble several natural water-bottoms examined in this study. The color and visible texture are similar and both are corrosive to aluminum.

3. The Ultracentrifugation and Extraction of Old Cultures of Fuel Isolates-Oxidizing Organisms

Large particles produced by microorganisms growing in jet fuel clog filters and do not sediment at 30,000 X gravity. A centrifuge was used to separate the particles from the whole medium so they could be analyzed. This apparatus can develop a centrifugal field in the order of 100,000 to 200,000 X gravity. Thus, if the particles had a specific gravity greater than the suspending medium, they would be sedimented downward; if their specific gravity were less, they would be floated upward.

Old cultures were centrifuged at 100,000 X gravity for 18 hours at 5°C. To minimize disturbance to the centrifuged materials, a brake was not applied and the centrifuge coasted to a stop. The centrifugation yielded a very small sediment, a deeply colored clear amber solution and a dark floating pellicular material.

The solution obtained by centrifugation was mixed with equal parts of hexane, benzene, diethyl ether, and chloroform. The aqueous phase of these solvent systems was adjusted to pH 1.5, 7.0, and 13. All solvents extracted a colored compound at the lowest pH, but little color formed in the organic phase at pH 7 and none at pH 13. The pH adjustment was accomplished with perchloric acid or sodium hydroxide.

An aluminum coupon of 7075 was placed in each system except those at pH 13. Control solutions in which water or acid were substituted for microbial culture were also prepared. The rate of corrosion in all cases was a strong function of hydrogen ion concentraction and depended on the solvent system used. Coupons with the culture extract corroded faster than the control.

The abundant floating pellicular material obtained in the ultracentrifuge was removed from the clear solution in a syringe. A part of this material was placed in an ether-water system and a part of it was suspended in water with an aluminum coupon. In the ether-water system, the pellicle distributed color both in the aqueous and organic phases. The pellicular material did not appear to cause the rapid corrosion of the alloy 7075. It is believed that the pellicle is a contaminant which contributes mostly to filter clogging while the clear but colored supernatant is active in stimulating aluminum corrosion.

G. The Decomposition of Aluminum Alloys in Systems Containing Water and Emissible Organic Solvents

Fuel tank corrosion has been associated with systems containing immiscible solvents. In the field, the water bottom and the jet fuel layer represent such a system. Also, in the field, the content of each phase may be largely unknown. The mineral content of the water bottom can come from various sources such as the leaching of salts from soils or from the displacement water used in transport and transfer of fuel. The organic content of jet fuels differs from refinery to refinery and batch to batch, but they still meet a definite operational specification.

To extract and purify microbial products from old cultures of fuel-grown organisms, an attempt was made to determine the corrosivity of such extracts made with different solvents using systems at different hydrogen ion concentrations. In these systems, corrosion took place in the aqueous phase of the system with hexane, benzene, diethyl ether, and chloroform. The extent of corrosion depended strongly on pH. In chloroform at pH 1.5, the decomposition of alloy 7075 took place both in the water phase and in the organic phase. This corrosion of aluminum was dramatic and has been reported to occur with chlorinated hydrocarbons. In about one week the standard aluminum coupon used in this study was entirely dissolved. The medium-chloroform system became violet in the chloroform phase and dark brown in the aqueous phase. With this system the same changes appeared at pH 7 but a somewhat reduced rate.

The appearance of color in the chloroform phase suggests the degradation of chloroform accompanying the dissolution of the aluminum alloy. It is theorized that the aluminum alloy in the organic phase was continuously depolarized through the reduction of chloroform to some colored product (Figure 25).

In these first studies solvent systems containing old culture medium again corroded more rapidly than did those with water alone. Corrosion was also observed at the water-air interface of systems with hexane and benzene. Corrosion at this interface has not been observed previously in this study.

These observations emphasize that the aluminum bar immersed in two immiscible solvents sets up in a concentration cell. These studies suggest that such electrical cells can affect oxidations and reductions of ambient organic solutes or solvents, and thereby cause the formation of new hydrocarbon molecules with accompanying aluminum corrosion.

The diagram below shows the chemical mechanisms tentatively believed to be operative in causing the observed corrosion.

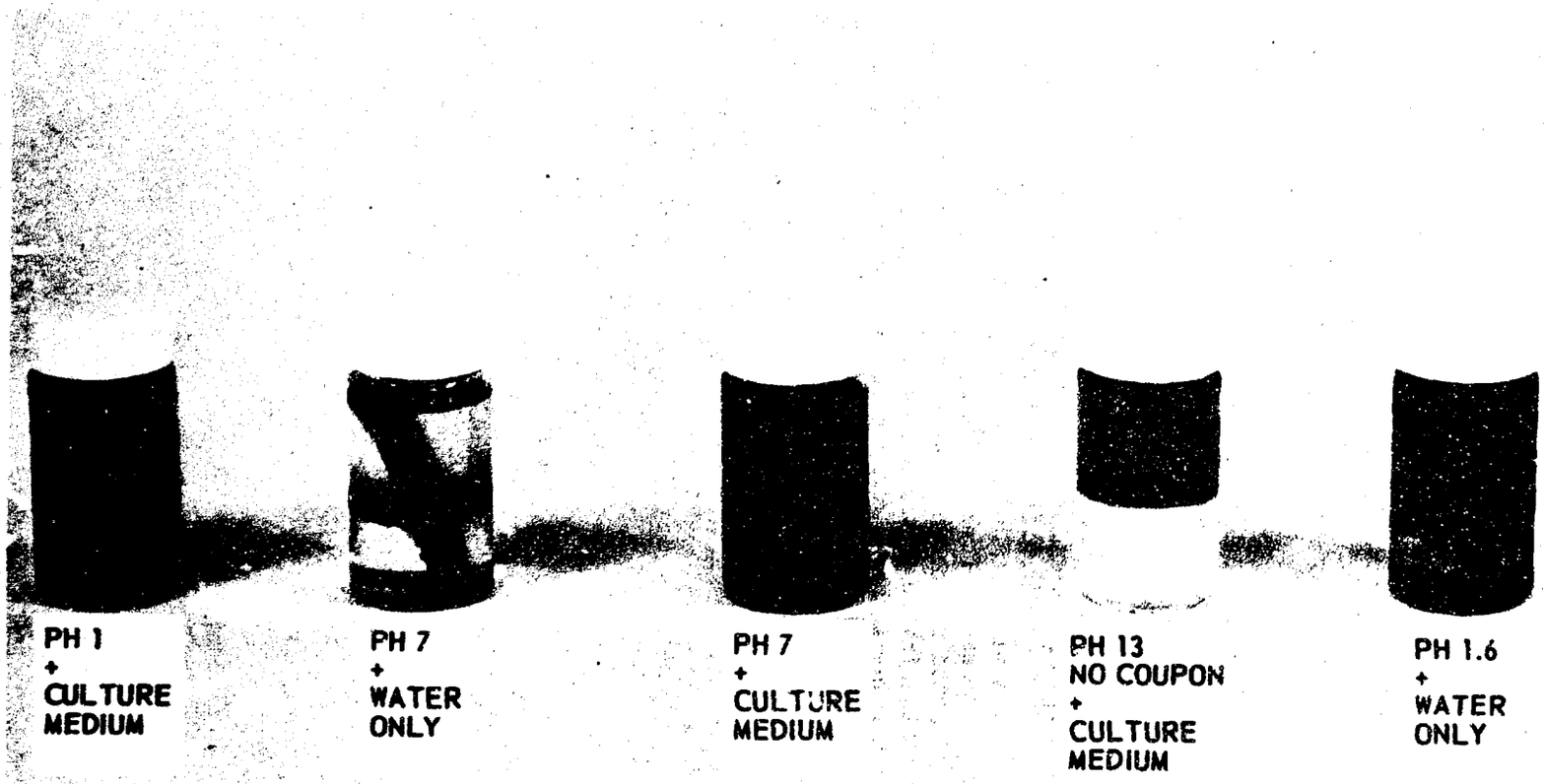
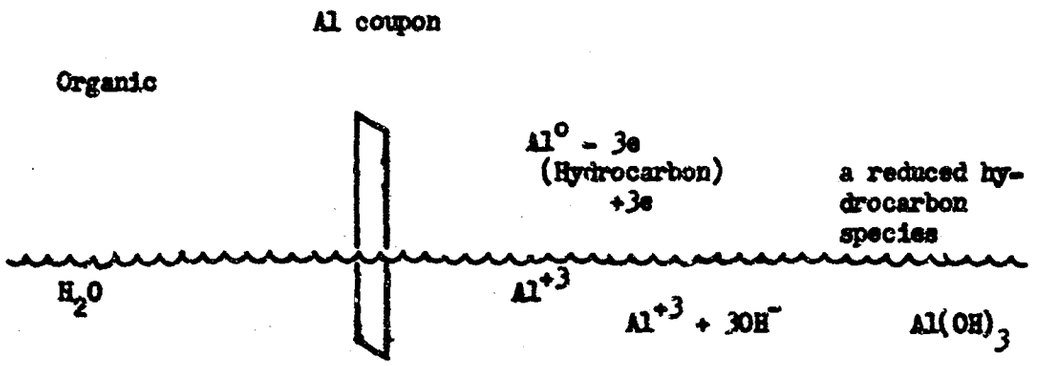
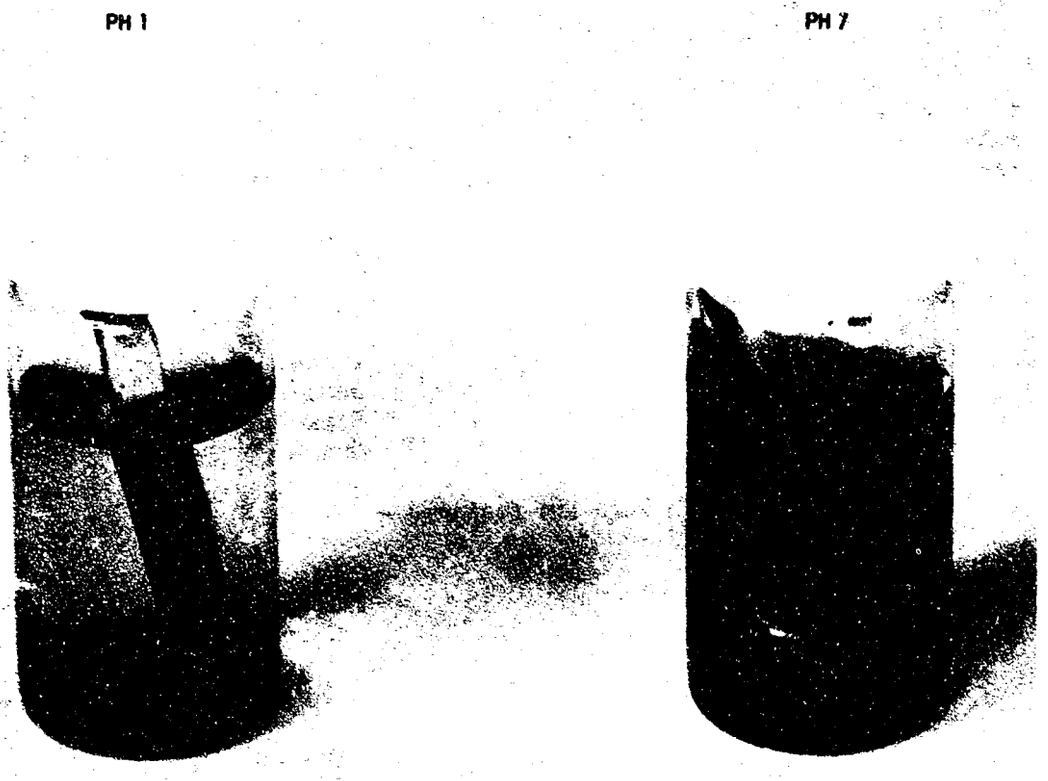


Figure 25. The Corrosion of Al° in Chloroform-Water Systems



The hydrocarbon undergoing reduction and perhaps subsequent oxidation could arise as a product of microbial metabolism. In chloroform solution a violet compound was produced. Stern and Uhlig[11] have noted the similarity of this compound to free radicals. In our study, as judged by color change, nitrated hydrocarbons also are readily decomposed in an aqueous solution containing an aluminum coupon (Figure 26). This decomposition accompanies aluminum destruction and may occur also with organic material produced by microorganisms.



THE ALLOY TESTED WAS 7075 AND THE 2,4 DINITROPHENOL CONCENTRATION WAS 10^{-4} MOLAR.

Figure 26. The Corrosion of Al° by 2,4-Dinitrophenol

H. The Microbial Deterioration of Sealant and Coatings (Sharpley Laboratories)

The principal effort during the past year has been to study the microbiological aspects of sealants and top coatings used in the manufacture of aircraft fuel tanks, and to investigate microbial concentration cell corrosion.

1. Preparation of Sealant and Coating Materials

A number of representative materials used in the industry were used to prepare films and are listed below. Additional identification of the materials may be obtained upon request to Air Force Aero Propulsion Laboratory (Attn: Jack R. Fultz), Wright-Patterson AFB, Ohio.

Generic description	Identification number
Buna-N type-coatings	
Sample 1	Buna-1
Sample 2	Buna-2
Sample 3	Buna-3
Sample 4	Buna-4
Polysulfide-type coatings and sealants - Chromate catalyzed	
Sample 1	Polysulfide-1
Sample 2	Polysulfide-2
Manganese dioxide catalyzed	
Sample 1	Polysulfide-3
Polyurethane type coatings	
Sample 1	Polyurethane-1
Sample 2	Polyurethane-2
Furan type	
Sample 1	Furan-1
Metal containing type	
Sample 1	Metal-1

The coating and sealant films were cast and cured according to the recommendations of the manufacturers. In some cases, coatings were cast on glass lightly coated with silicone. The polymerized films were subsequently removed for further testing. Films were also cast on aluminum and steel coupons as well as nylon fabric.

2. Utilization of Sealants and Coatings for Growth

Top coatings and sealants were tested to determine whether they could serve as a carbon and nitrogen source for fuel isolates. There were sufficient contaminants in the carbon and nitrogen-free media to allow microbial growth and confuse laboratory results, even though the purest available chemicals were used. To minimize the effects of extraneous carbon and nitrogen contaminants, batches of media in Fernbach flasks were inoculated with a mixed culture of fuel isolated microorganisms and cultured for 3-5 days at 28°C on a rotary shaker. The cells were removed by filtration through an 0.3μ pore size membrane filter. Although time-consuming, this technique reduced microbial growth in controls to a reasonable level.

The nitrogen and carbon-free media used in this work were as follows:

Carbon-free medium

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.4	g
Calcium chloride ($CaCl_2$)	0.02	g
Potassium phosphate, dihydrogen (KH_2PO_4)	2.0	g
Potassium phosphate, monohydrogen (K_2HPO_4)	2.0	g
Ammonium phosphate ($NH_4H_2PO_4$ - $(NH_4)_2HPO_4$-1:1)	0.599	g
Distilled water, q.v.	1000	ml

Nitrogen-free[1] medium

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.4	g
Calcium chloride ($CaCl_2$)	0.02	g
Potassium phosphate, dihydrogen (KH_2PO_4)	2.0	g
Potassium phosphate, monohydrogen (K_2HPO_4)	2.0	g
Dextrose	1.0	g
Distilled water, q.v.	1000	ml

(1) Label analyses indicate 7.8 ppm N as contaminants. Chemical analysis gave a mean of 4.03 mg N/liter.

Free films were prepared and extracted in boiling distilled water to remove the water soluble nutrients. Fernbach flasks were used which contained 200 ml of carbon-free media and 10 gm of pieces of film. Cultures were determined by direct count using a Petroff-Hauser chamber and cell viability verified by plate count.

The results in Table 11 show that up to 10-fold increases in microbial growth over the control were obtained with:

Polysulfide-3
Buna-4 extracted
Buna-2 extracted
Furan-1 extracted
Polysulfide-2

Greater than 10-fold increases in growth over the control were obtained with:

Buna-3 not extracted
Polyurethane-1
Metal 101
Buna-4 not extracted
Buna-2 not extracted
Furan-1 not extracted
Polysulfide-2 not extracted
Buna-1

The determination of microbial growth in a nitrogen-free medium was similar to the method outlined for the carbon-free medium. The results shown in Table 12 were as follows:

Growth equal to or greater than the control was obtained when every extracted coating was combined with the exhausted nitrogen-free medium.

Less than 2-fold increase in growth over the inoculated control without test substrate was obtained with:

Buna-3
Polyurethane-2 not extracted
Polyurethane-1
Buna-2 extracted
Polysulfide-1
Buna-4
Buna-2 not extracted

TABLE 11

GROWTH OF BACTERIA ON CARBON-FREE MEDIA

	Carbon-Free Media Bacteria/ml/x10^7					
	1st Substrate		2nd Substrate		3rd Substrate	
	Ext.	Not Ext.	Ext.	Not Ext.	Ext.	Not Ext.
Polyurethane-2	31	31	1.2	1.4	.27	.34
Buna-3	32	52	3.1	23	2.3	9.3
Polyurethane-1	50	48	12	15	7.2	8.1
Metal-1	52	67	44	71	12	91
Control	22		.88		.25	
Polysulfide-3	9.1	14	.94	6.1	1.4	1.05
Buna-4	11	33	3.1	77	2.0	23
Buna-2	16	8.9	3.5	22	0.7	15
Control	7.0		0.7		.27	
Polysulfide-1	6.1	6.6	1.1	1.3	1.2	1.6
Control	3.4		.97		.88	
Furan-1	18.3	91.9	4.1	13	.74	6.1
Polysulfide-2	22.5	22.2	5.2	8.0	.96	3.3
Polyurethane-2	35.5	27.5	2.5	.64	.44	.39
Buna-1	20.3	12.8	2.2	7.2	1.7	5.3
Control	14.8		.2		.13	

Notes:

1. All counts are direct microscopic courts using a Petroff-Hauser chamber. Cell viability confirmed by plate count. Incubation 4-7 days, agitated at 28°C.

2. 2nd and 3rd substrates are adapted cells. Evaluations on basis of 3rd substrate. See text for further definitions.

3. "Ext" in the sub-headings indicates that the coating had been extracted in boiling distilled water after polymerization and curing. "Not Ext" indicates the coating was used as is.

TABLE 12

GROWTH OF BACTERIA ON NITROGEN-FREE MEDIA

	Nitrogen-Free Media Bacteria/ml/10^7					
	1st Substrate		2nd Substrate		3rd Substrate	
	Ext.	Not Ext.	Ext.	Not Ext.	Ext.	Not Ext.
Polyurethane-2	38	31	3	2.8	4.7	2.0
Buna-3	32	53	4.7	4.0	1.9	2.6
Polyurethane-1	37	39	2.1	1.7	2.4	2.6
Metal-1	100	130	45	100.	18	55
Control	30		1.8		2.0	
Polysulfide-3	13	29	6.5	7.2	5.4	5.2
Buna-4	11	11	4.5	2.0	3.7	3.1
Buna-2	7.2	15	3.1	2.0	2.0	2.7
Control	20		2.2		2.4	
Polysulfide-1	8.1	9.2	2.75	2.8	1.8	1.1
Control	5.8		1.4		1.0	
Furan-1	26.3	20	5.3	6.1	1.0	.58
Polysulfide-2	26.7	30.6	4.5	4.4	.52	.66
Polyurethane-2	28.3	30.2	2.8	4.7	.47	.63
Buna-1	10.9	19.1	9.4	11	.69	1.1
Control	25.6		.91		.22	

Notes:

1. All counts are direct microscopic counts using a Petroff-Hauser chamber. Cell viability confirmed by plate count. Incubation 4-7 days, agitated at 28°C.

2. 2nd and 3rd substrates are adapted cells. Evaluations on basis of 3rd substrate. See text for further definitions.

3. "Ext" in the sub-headings indicates that the coating had been extracted in boiling distilled water after polymerisation and curing. "Not Ext" indicates the coating was used as is.

Up to 4-fold increase in growth was obtained with the following:

Polyurethane-2 extracted
Polysulfide-3
Furan-1
Polysulfide-2
Buna-1

A great increase in growth was recorded for:

Metal-1

A second method was explored for determining whether coatings accelerated or inhibited growth.

Water-soluble materials present in coatings may either stimulate or inhibit the growth of bacteria. The detection of such materials is of importance when evaluating growth tests and also for background information concerning toxic additives.

The procedure used was to polymerize and cure the coating. The coating was then minced and 5 grams extracted for 30 minutes in 100 ml boiling distilled water. Discs were cut from the cast films and assayed both before and after extraction. Filter paper assay discs were saturated with the water extract for testing.

Melted and cooled TGE agar was inoculated with the mixed bacterial culture and poured into petri dishes. After solidification, extracted and non-extracted discs of coatings and filter paper discs saturated with the water extracts were placed on the surface of the agar. All tests were run in triplicate. Inhibition was judged to be present on the basis of a clear zone surrounding the test disc. Stimulation was recorded if a zone of increased growth surrounded the disc, or if there was a slight zone of inhibition surrounded by a zone of stimulation.

Inspection of these data show the following:

a. Four Buna-N type compounds were tested and the water extracts from three of the compounds stimulated bacterial growth. The fourth material contains inhibitory substances not defined in these tests.

b. Chromate-polymerized-polysulfide sealant, Polysulfide-2, was inhibitory in these tests.

c. The manganese-dioxide-polymerized polysulfide sealant stimulated growth.

d. Polyurethane type topcoating material shows some stimulation of growth in these tests.

e. Water extracts from furan-type materials are slightly inhibitory.

3. Microbial Deterioration of Coated Steel and Aluminum Coupons

Aluminum and steel coupons were coated with a number of sealants and top coatings. These coupons were then incubated in TS-11 containing JP-4 and microorganisms for 4 to 5 months. Before initiating this study it was necessary to determine whether TS-11 supported better growth of "wild" fuel isolates than other conventional media, and whether the medium was corrosive.

In general, it was found that TS-11 was a more suitable growth medium for microorganisms isolated from fuel than any other medium currently available. TS-11 was used in our work at this laboratory and has the following composition:

$MgSO_4 \cdot 7H_2O$	0.4	g
$CaCl_2$	0.02	g
KH_2PO_4	2.0	g
K_2HPO_4	2.0	g
NH_4NO_3	0.1	g
$CuSO_4 \cdot 5H_2O$	0.027	g
Fe powder	4.0	g
Distilled water	1000	ml

TS-11 was also tested for its corrosive effects on aluminum coupons (7075) by visual appearance and weight loss determinations. In general, chemical corrosion with TS-11 was slightly greater than Bushnell-Haas, although not as great as that caused by tap or distilled water.

The inspection of the coating panels was quite detailed. Each panel was removed from the fuel-water medium, dried, and inspected for obvious film failure. The panels were then scanned at 20X to determine small holidays and flaws. Finally, an attempt was made physically to strip the coating from the substrate and thus determine the degree of adherence. Where it was possible to strip the coating cleanly, the underlying metal was examined for corrosion associated with flaws in the coating.

The majority of "sterile" controls contained microorganisms at the end of the incubation period. These were quite possibly introduced with the coatings, since it was not possible to completely sterilize them, or during the previous inspections. In most cases, the growth in the control is much less than in the inoculated series. Sterile controls were maintained with Furan-1 and Buna-3. (See Table 13).

TABLE 13

OBSERVATION OF COATED PANELS

Coating	Peeling	Blistering	Color Extraction	Corrosion	Swelling
Metal-1	Severe	None	None	-	None
Furan-1	None	Severe(Steel) Minor(Al)	None	-	Present
Buna-1	None	Present	Severe	Pitting	Present
Polysulfide-1	None	Present	Present	Edge (steel)	Present
Polyurethane-2	None	None	None	None	None
Buna-4	None	Present	Present	-	Present
Buna-3	None	None	Present	-	None
Polyurethane-1	None	None	None	None	None
Buna-2	None	Present	Present	-	None

Remarks: (1) Coatings were applied to both steel and aluminum coupons. Where there was a difference, it is so noted in results.

(2) It was not possible to clearly remove all coatings for observation of underlying corrosion. A dash indicates no readings were made.

Swelling has frequently been noted in Buna-A type coatings as well as in other types of polymers. Careful microscopic examination of swelled coatings indicates a very sharp line of demarcation. The coatings appear to lose adherence under these circumstances, but little damage is noted visually. The microscopic examination indicates that swelling may be symptomatic of more severe coating damage.

It has been often stated that corrosion will occur under holidays in coatings. Observations in the field have substantiated the occurrence of pits under flaws in coatings, but, to our knowledge, this has rarely been documented in the laboratory. During the course of these experiments, it was possible to demonstrate corrosion pits immediately under holidays (Figure 27).

Figure 27. Photomacrograph of Coated Coupon with Coating Peeled Back to Show the Holiday and Corresponding Pit in the Metal

Heavy corrosion was observed under coatings that were severely blistered. A photomicrograph of a blistered panel which demonstrates the corroded areas under the blisters, as well as the pits so formed, is shown in Figure 28.

The formation of small bubbles in top coatings is very common and is considered undesirable. However, little attention has been paid to them if the bubbles were small in size and few in number. Our observations indicate that bubbling may be a more severe problem than is generally recognized. In many cases, the bubbles apparently break down and form pinholes in the liquid phase. Little or no effect was noted in the vapor phase. Very small pinholes were documented in Buna-N coatings that bubbled severely and it was the microscopist's opinion that the pinholes resulted from bubble breakdown.

4. Modified Hazzard Tests

Nylon net was coated with various polymers under investigation using a modification of the procedure recommended by Hazzard.[12] The results of the series has confirmed the other data reported here. Failure, as demonstrated by growth, was obtained with Buna-N, Furan, and Zincilate. The polyurethane and polysulfide coatings were not penetrated by microorganisms. Work was discontinued on the Hazzard tests since it proved difficult to obtain a satisfactory coating film.

5. Soil Burial Tests

One of the techniques planned was to obtain adapted cultures from coatings subjected to soil burial in the presence of JP-4. The work on coatings was terminated before isolations of the microflora were made, but the following observations were made and they are thought to be of interest.

Excellent mold growth has been obtained on the soil-manure-water-JP-4 mixture. The fungi are mixed but large numbers of an actinomyces grew, along with numbers of Alternaria, and probably, Trichothecium.

Empirically, it appeared that the Buna-N type and Furan-1 type coatings may have lost plasticizer, since they are quite brittle. The polysulfide and polyurethane do not seem to have been affected.

Fungal growth has been demonstrated on both the Furan-1 and the Buna-N coatings by microscopic examination. It is not known whether the hypha penetrated the coating. However, the microscopic appearance is very similar to fungal growth on other molded coatings that are known to be penetrated by fungal mycelia.

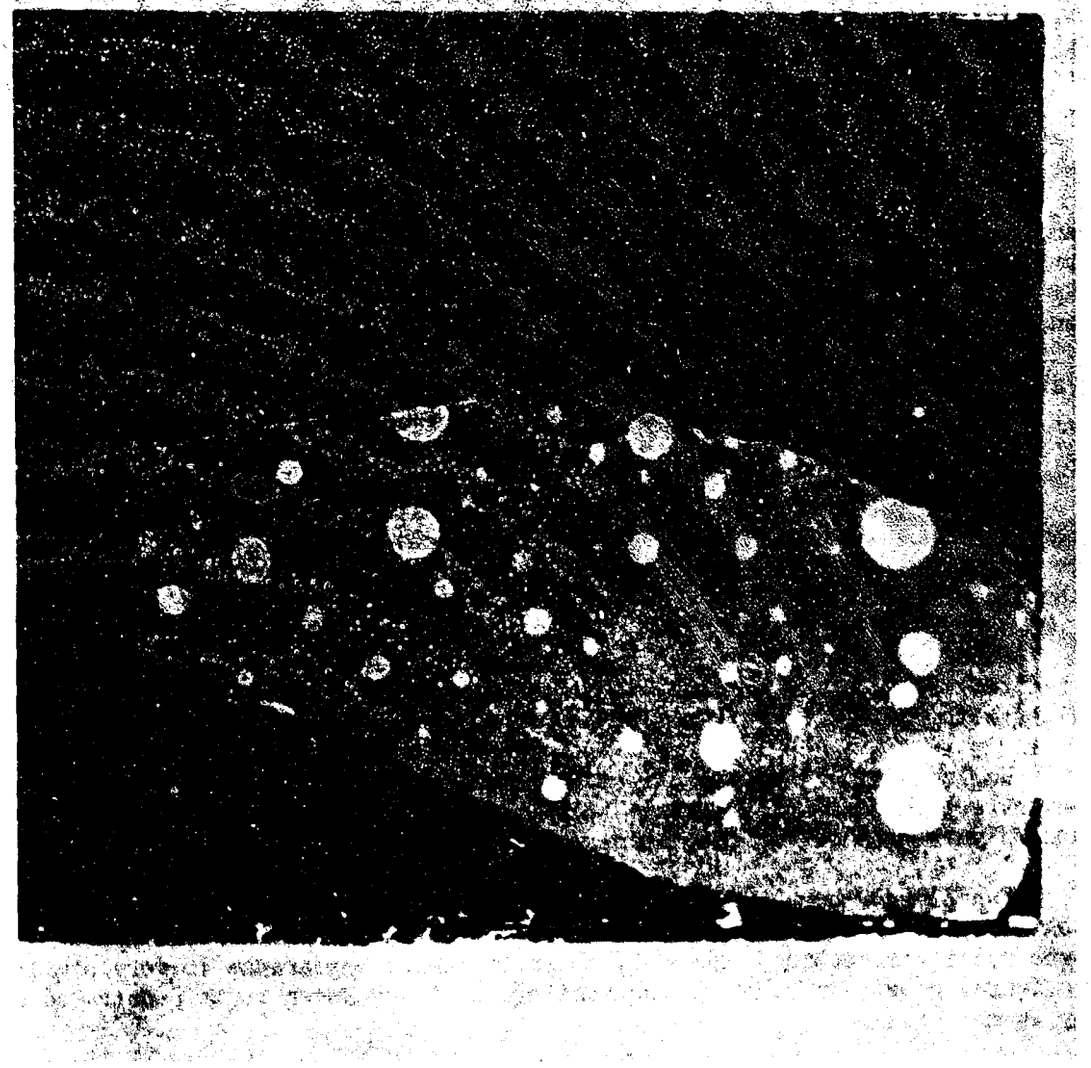


Figure 28. Photomacrograph of Coated Coupon with Severe Blistering. Blistered Coating has been Stripped to Show Corrosion

I. Microbial Concentration (Sharpley Laboratories)

Work has been initiated to characterize the mechanisms by which microorganisms may cause pitting corrosion.

1. Inverted Test Tube Technique

This method consists of cementing a small test tube to an aluminum coupon by means of epoxy glue. The test tubes contained 2.5 ml of Bushnell-Haas medium, 0.5 ml of sterile kerosene. Inoculum used in these experiments consisted of:

a. Cladosporium sp.
b. Pseudomonas sp.
c. Fusarium sp.
d. Paecilomyces varioti

The 7075 aluminum coupons were cleaned by dipping them in a 0.5 percent NaOH solution at 50°C, followed by a water rinse and immersion in 1:1 HNO_3. The cleaned coupons were dried in acetone. Following inoculation, the coupons were cemented to the test tubes and allowed to dry overnight. The test tubes were then inverted for incubation at 28°C for four months.

This technique yielded essentially no results that are worth including in this report. Difficulty was experienced in obtaining a sound glue joint, and the culture medium leaked from a number of inverted test tubes over the period of incubation. Examination of the remaining tubes with sound glue joints indicated little or no corrosion of the aluminum coupons. There was little or no change in the pH or total titratable acidity of the culture media which would reflect the metabolic activities of the microorganisms. In retrospect, it now appears that there was probably insufficient oxygen for good growth of the microorganisms. Also, since there was no oxygen balance, an aerobic concentration cell could not be formed. This work has been discontinued.

2. Accumulation of Copper by Fungi

It is known that microorganisms, especially fungi, incorporate various metals in their cell structure. It might well be that one mechanism for the microbial corrosion of aluminum is the entrainment of metals into cell tissue that are corrosive to aluminum. Various experiments to test this possibility are described as follows.

Preliminary work using copper has been completed using this theory as a basis for the experimental design. Hormodendrum was used as one test fungus because of its common occurrence in fuel, and Pullularia since it is known to accumulate appreciable amounts of metal and is a very common organism.

A simple peptone medium was used for culture in Fernbach flasks agitated at 160 rpm at 28°C. Copper as copper sulfate was added in concentrations of 0, 10 and 50 ppm. The concentrations were calculated as metallic copper. Some difficulty was experienced adapting the cells to growth in the higher concentrations of copper, since it is quite toxic to fungi, but satisfactory growth was finally obtained by serial transfer on ascending concentrations of copper.

After growth was obtained in 3-4 days, the cells were harvested by centrifugation at 2000 rpm for 30 minutes. The cells were then washed with distilled water, centrifuged again, and taken to dryness on a membrane filter. They were again washed a number of times on the filter with small amounts of distilled water to remove copper-containing medium. Final yield was 1.5-2.5 grams of wet mycelia.

The wet mycelia was dried by vacuum until free moisture was removed. Although some work was done on completely dried mycelia, it was found that dried material was very difficult to handle, hence the experiments reported here were made with moist cells.

The mycelial mass was separated into several portions. Chemical analysis for copper was made on one portion and the remaining material was placed as a small mass on the surface of aluminum coupons, as described subsequently.

The collected mycelia were analyzed for copper using bis (2-hydroxyethyl) dithiocarbamate. Weighed portions of the mycelial mass were digested with sulfuric acid catalyzed with selenium. The liquid was then neutralized with caustic, and color developed in an appropriate portion with the chelating agent. Some difficulty was experienced with masking precipitations, presumably of selenium, but this problem was solved by extracting the chelate from the water solution with iso-amyl alcohol. Estimations of copper were made on the basis of a standard spectrophotometric curve prepared from known copper solutions at 435 mu. Copper content of the mycelia examined is shown in Table 14.

The analytical data are only approximate since the moisture content is not known, but they are sufficient to indicate an appreciable accumulation of copper. The data are not yet complete, as indicated in the table.

TABLE 14

COPPER CONTENT OF HARVESTED FUNGAL CELLS

Copper concentration in media (ppm)	Copper (ppm) found by analysis of mycelia			
	Hormodendrum		Pullularia	
	Test 1	Test 2	Test 1	Test 2
0	Less than 0.05		Less than 0.05	
10	2.5	2.25	-	3.5
50	2.5	4.4	4.0	-

Each of the pellets from the Hormodendrum cultures were placed on the surface of chemically cleaned 2x2 inch type 2024 aluminum coupons. The coupons were then placed in sealed petri dishes containing moist filter paper to prevent drying of the slime masses.

Severe pitting corrosion was obtained after 60 days using Hormodendrum. The corrosion was considerably more severe with those mycelial pellets containing copper. The pit depths were estimated and the diameter was measured on the microscope. (Table 15.)

3. Accumulation of Iron by Fungi

Six Fernbach flasks containing 500 ml of a 1.0 percent peptone medium were sterilized. A solution of $FeCl_3 \cdot 6H_2O$ was added in such a way that two flasks contained 10.0 ppm of $FeCl_3 \cdot 6H_2O$ calculated as Fe and two flasks contained 50 ppm Fe. Two flasks to which no iron was added served as controls. Pullularia pullulans was added to three Fernbach flasks containing 0.0, 10.0 and 50.0 ppm of Fe respectively. A Cladosporium isolate was inoculated in a similar fashion into three flasks.

The six flasks were placed on the rotary shaker for three days at an incubation temperature of 28°C.

The fungal cells were harvested by centrifuging the contents of each flask. The supernatant culture medium was poured off and the residue of fungal cells were taken up in ethyl alcohol and filtered. The harvested residue was dried in an oven at 75°C overnight.

Small portions of the harvested fungal residue are placed on clean 7075 aluminum coupons which are placed in petri dishes containing wet filter paper and sealed with rubber bands. This procedure provides an incubation chamber which will maintain a relative humidity of 95 to 100 percent for an extended period of time.

The harvested residue of fungi was analyzed for iron. Weighed amounts of dried fungal cells were digested by conventional Kjeldahl methods. Iron determinations were made by reacting portions of the neutralized Kjeldahl digest with phenanthroline and measuring the percent transmission at 510 mμ on a Beckman spectrophotometer. This work is in progress and there is no data yet available.

4. Corrosion of Aluminum by Metals Accumulated in Nonbiological Binders

It was thought that if microorganisms can accumulate metals on or within their cell structures that are corrosive to aluminum, it should be possible to verify this theory by incorporating metals in nonbiological binders and compare the degree of corrosion. Hence, the experiment reported here was prepared and designed to parallel the work on metal accumulation by fungi.

TABLE 15

PITTING CORROSION RESULTING FROM HORMODENDRUM GROWN WITH COPPER

Control	Small scattered pits are present. Pits are fairly shallow, steep sided and small in diameter. Pits range in the order of 30-40μ deep and less than a millimeter in diameter.
Mycelia Containing Copper (10 ppm series)	Pitting is more extensive than control, although of the same type. Pits range more than a millimeter in diameter and up to 100μ deep.
Mycelia Containing Copper (50 ppm series)	Much more severe than the control or low concentration of copper. Pits are 3-4 mm in diameter and greater than 150μ deep. There is typical undermining characteristic of intergranular corrosion.

Solutions of $CuSO_4 \cdot 5H_2O$, $FeCl_3 \cdot 6H_2O$ and $ZnSO_4 \cdot 7H_2O$ were added to 3.0 percent agar in such amounts that the following concentrations were obtained, based on the cation of each solution.

Ppm of Cation in Agar

0.0
5.0
10.0
15.0
25.0
50.0
100.0
1000.0

The melted agar containing the neutral salts solutions in various concentrations were poured into petri dishes and allowed to harden. Agar plugs were obtained from the petri dishes and placed on cleaned aluminum coupons. Each concentration of each salt was replicated three times.

The aluminum coupons were cleaned by immersion in 5.0 percent NaOH solution at 50°C. followed by a water rinse and immersion in 1:1 HNO_3. The cleaned coupons were stored under acetone until use.

The experiment is in progress and hence no data has been acquired. The agar plugs are translucent and the indications are that appreciable corrosion is occurring.

7 REFERENCES

1. Blanchard, G.C. and Goucher, C.R., "Mechanism of Microbiological Contamination of Jet Fuel and Development of Techniques for Detection of Microbiological Contamination." January 1964 APL-TDR-64-70, Part 1 AF Aero Propulsion Laboratory Research and Technology Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio.

2. Takahaski, et al., "Tripase Formation by Microorganisms from Hydrocarbons," Agricultural and Biological Chemistry, 27, 1963.

3. Yamada, et al. "Studies on the Utilization of Hydrocarbons by Microorganisms, Part 1, Isolation of Amino Acid-Producing Bacteria from Soil." Agricultural and Biological Chemistry, 27, 1963.

4. Bergey's Manual, Determinative Bacteriology by R. S. Breed, E. G. D. Murray, M. R. Smith and 94 contributors, 7th edition, Wms. and Wilkins Co., Baltimore, 1957.

5. Blanchard, G. C. and Goucher, C. R., "Aluminum Corrosion by Microbial Cultures," Developments in Industrial Microbiology, Vol. 6, 1964.

6. Burchfield, H. P., Storrs, E. E., Biochemical Applications of Gas Chromatography, Academic Press, New York, 1962.

7. Metcalfe, L. D., and A. A. Schmitz, "Esterification Procedure," Anal. Chem., 33, 363-4 (1961).

8. Bobbitt, J. M., Thin Layer Chromatography, Reinhold Publishing Co., New York (1963).

9. "Three-Minute Esterification of Fatty Acids, Esterification Procedure," 1963 Bulletin: Applied Science Laboratories Inc., State College, Pa.

10. Gholson, R. K., et al., "Hydrocarbon Oxidation by a Bacterial Enzyme System," Biochemistry, 2, 1155 (1963).

11. Stern, M., and Uhlig, H. H., J Electrochem. Soc. 100 p. 543 (1953).

12. Hazzard, G. F. and E. C. Kuster, "Fungal Growths in Aviation Fuel Systems. Part 2. Test Methods," (Rept. No. 252) 14 p. Defence Standards Lab (Australia) Dec. 1962. Defense Documentation Center No. AD-298-174.

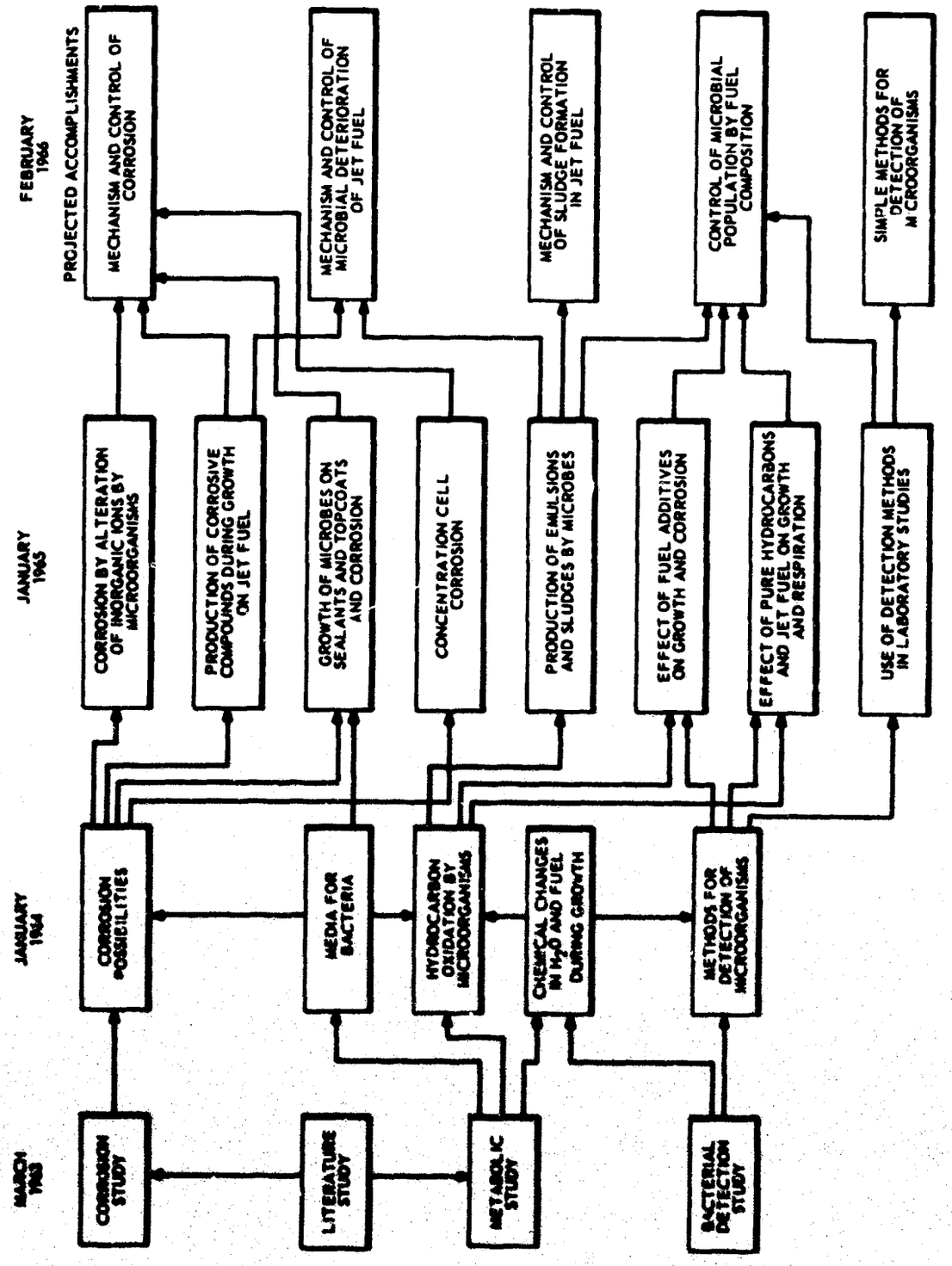


Figure 29. Flow Diagram of Accomplishments and Future Plans